Predicting outcome in ulcerative colitis: Clinical and genetic determinants of disease susceptibility and behaviour

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For Lynn, Millie and Charlie

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Contents:

Abbreviations

ABC	ATP-Binding Cassette
ANCA	Anti-Neutrophil Cytoplasmic Antibodies
APC	Antigen Presenting Cells
ASCA	Anti Sacchromyces Cerevesiae Antibodies
AUC	Area Under Curve
AZA	Azathioprine
CARD	Caspase Recruitment Domain Family
CD	Crohn's Disease
CEPH	Centre D'Etude du Polymorphisme Humain
CI	Confidence Interval
CS	Corticosteroids
Cyp450	Cytochrome P450
DLG5	Drosophila Large Gene 5
GR	Glucocorticoid Receptor
GWAS	Genome wide association studies
HLA	Human Leukocyte Antigen
IBD	Inflammatory Bowel Disease
IL	Interleukin
IV	Intravenous
LD	Linkage disequilibrium
MDP	Muramyl Dipeptide
MDR1	Multidrug resistance 1

MHC	Major Histocompatibility Complex
MRNA	messenger RNA
MSF	Mean stool frequency
MYOB9	Myosin IX
NFKB	Nuclear factor kappa-beta
OCTN	Organic Cation Transporter
OR	Odds ratio
PgP	P-glycoprotein
PBS	Phosphate Buffer Solution
PLEM	Partition Ligation Expectation-Maximization
ROC	Receiver Operator Characteristics
Rs	reference sequence
SNP	Single nucleotide polymorphism
PCR	Polymerase chain reaction
PXR	Pregnane X-Receptor
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
tSNP	Tagging single nucleotide polymorphism
TPN	Total parenteral nutrition
UC	Ulcerative Colitis
5-ASA	5-aminosalicylate
6-MP	6-mercaptopurine

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Statement of originality

The investigations described in this Thesis all represent original work. Gwo-Tzer Ho in Edinburgh, between December 2001 and December 2004, carried out all work. The Lothian Research and Ethics Committee (LREC) approved the studies from this thesis and written consent was obtained from all patients.

1. Drs. Nicole Soranzo and Sarah Tate contributed jointly to the selection of haplotype tagging single nucleotide polymorphisms for the Multidrug resistance 1 (MDR1), Pregnane-X receptor (PXR) and Multidrug resistant protein 3 (ABCC3/MRP3) genes. The resequencing of candidate genes in this thesis was carried out in Professor David Goldstein's laboratory, University College London. Access to the CEPH Caucasian trios DNA was kindly provided by Professor David Goldstein. Dr. Albert Tenesa (MRC Human Genetics Unit, University of Edinburgh) provided practical assistance and designed computer programs for haplotype analysis and linkage disequilibrium calculations.

2. Neel Shah, Terence Ting, Daniel Gilpin, Priscilla Chiam, Sam Murray, Danda Seddon and Roddy Gunn (all University of Edinburgh medical students provided assistance in case note review).

3. Genomic DNA sequencing was also performed in the MRC Human Genetics Unit, University of Edinburgh. TaqMan genotyping was undertaken within the Wellcome Genetics Core, Western General Hospital, Edinburgh.

Abstract

Ulcerative colitis (UC) and Crohn's disease (CD), collectively known as inflammatory bowel disease (IBD) are common, chronic inflammatory disorders of the intestines. The pathogenesis and subsequent clinical course of IBD remain unclear; although, inter-relating factors such as environment triggers, dysregulated immune response, defective gut barrier defence, variability of drug response and genetic susceptibility all contribute. The current work described in this thesis involves a series of clinical and genetic studies investigating their respective roles in determining susceptibility and course in UC. Firstly, the importance of corticosteroid resistance/dependence as a co-factor in disease progression was studied in a detailed 5-year inception cohort (1998-2003). This study demonstrated remarkable concordance with older series and paediatric cohorts suggesting a defined phenomenon within the innate response to corticosteroids. Secondly, a risk score to stratify the likelihood of response to standard medical therapy (high dose corticosteroids) identifying 3 distinct risk groups - low, intermediate and high risk; based on 167 consecutive patients (largest series studied) with acute severe UC was developed. A novel robust model was formulated with a sensitivity/specificity of 85% and 75% respectively to predict failure of medical therapy. These initial studies have critically provided the assimilation of highly accurate phenotypic and follow-up data, permitting very detailed statistical analyses to be performed in subsequent genetic studies. The multidrug resistance gene (MDR1gene-encoding P-glycoprotein 170, an epithelial efflux transporter protein) was studied in detail due to its potential role in conferring susceptibility (based on knock-out animal models and genomic position within IBD locus of susceptibility) and its function in corticosteroid resistance. In this study, allelic

variations of the MDR1 gene were found to be implicated in disease susceptibility in UC, in particular extensive and severe disease subphenotype (p=0.003, OR 2.64 and T-allele, p=0.009, OR 1.70. In this study, bi-directional haplotypic contribution to susceptibility in UC (with protective and susceptible haplotypes) was observed. This led to a more rigorous analysis of this gene by the application of the novel 'gene-wide' haplotype tagging approach, confirming a more significant association with UC ($p=4.22x10^{-7}$) but not CD (p=0.22). The strongest association was with the sub-phenotype; extensive UC ($p=1.7 X 10^{-7}$), and critically dependent on one tSNP rs3789243 ($p= 3.2 x 10^{-7} - 3.6 x 10^{-12}$). This catalysed further rational approaches to study the genetic variations of the genes involved in xenobiotic-metabolism and epithelial transport such as the ATP-binding cassette (ABC) efflux transporters and respective transcriptional regulators in our cohort. Further significant and replicable association of haplotypic variations of the ABCC3/MRP3 gene (also involved in epithelial barrier defence) with IBD (p=0.00004, 1200 IBD and 700 controls) was also shown. The characterisation of these genes in a hitherto unknown pathway regulated by key transcriptional regulators such as Pregnane-X receptor (PXR) has now implicated this novel class of transport proteins as important regulators of mucosal defence and as critical in determining susceptibility to inflammatory bowel disease.

Awards

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Publications

1. **Ho G.T.**, Nimmo ER, Tenesa A, Fennell J, Drummond H, Mowat C, Arnott ID, Satsangi J. Allelic variations of the multidrug resistance gene determine susceptibility and disease behavior in ulcerative colitis. Gastroenterology 2005 Feb; 128(2):288-96.

2. **Ho G.T.**, Soranzo N, Nimmo ER, Tenesa A, Goldstein DB, Satsangi J. ABCB1/MDR1 gene determines susceptibility and phenotype in ulcerative colitis: discrimination of critical variants using a gene-wide haplotype tagging approach. Human Molecular Genetics 2006 Mar 1;15 (5):797-805.

3. **Ho G.T.**, Soranzo N, Tate SK, Nimmo ER, Drummond HE, Tenesa A, Arnott ID, Goldstein DB, Satsangi J. Lack of association of the pregnane X receptor (PXR/NR1I2) gene with inflammatory bowel disease: parallel allelic association study and gene wide haplotype analysis. Gut 2006 Nov; 55(11):1676-7.

4. **Ho G.T.** Soranzo N., Tenesa A., Nimmo E.R., Drummond H.E., Goldstein D.B. and Satsangi J. Gene-wide haplotypic analysis demonstrates association between ATP-Binding cassette 3/ Multi Resistance Protein 3 (ABCC3/MRP3) gene and inflammatory bowel disease. Gastroenterology 2007 (*submitted*).

5. Noble CL, Nimmo ER, Drummond H, **Ho G.T.**, Tenesa A, Smith L, Anderson N, Arnott ID, Satsangi J. The contribution of OCTN1/2 variants within the IBD5 locus to disease susceptibility and severity in Crohn's disease. Gastroenterology 2005 Dec; 129 (6):1854-64.

6. **Ho G.T.**, Chiam PS, Drummond H, Loane J, Arnott ID, Satsangi J. The efficacy of corticosteroid therapy: analysis of a 5-year UK inception cohort. Alimentary Pharmacology and Therapeutics 2006 Jul 15;24 (2):319-30

7. **Ho G.T.**, Mowat C, Goddard CJ, Fennell JM, Shah NB, Satsangi J. Predicting the outcome of severe ulcerative colitis: development of a novel risk score to aid early selection of patients for second-line medical therapy or surgery. Alimentary Pharmacology and Therapeutics 2004 May 15;19(10):1079-87.

8. **Ho G.T.**, Moodie FM, Satsangi J. Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? Gut 2003 May;52(5):759-66.

9. **Ho G.T.**, Lees C, Satsangi J. Pharmacogenetics and inflammatory bowel disease: progress and prospects. Inflammatory Bowel Disease 2004 Mar; 10(2):148-58.

10. **Ho G.T.**, Gaya DR, Satsangi J. Multidrug resistance (MDR1) gene in inflammatory bowel disease: a key player? Inflammatory Bowel Disease 2005 Nov;11(11):1013-9.

11. **Ho G.T.**, Seddon AJ, Therapondos G, Satsangi J, Hayes PC. The clinical course of ulcerative colitis after orthotopic liver transplantation for primary sclerosing cholangitis: further appraisal ofimmunosuppression post transplantation. European Journal of Gastroenterology and Hepatology 2005 Dec;17 (12):1379-85.

12. Brady RR, Collie MH, **Ho G.T.**, Bartolo DC, Wilson RG, Dunlop MG. Outcomes of the rectal remnant following colectomy for ulcerative colitis. Colorectal Dis. 2007 Feb 14; (Epub ahead of print).

13. Van Limbergen J, Russell R.K., Nimmo E.R., **Ho G.T.**, Satsangi J and Wilson D. The genetics of innate immunity in inflammatory bowel disease. Inflammatory Bowel Disease 2006 Dec 19 (Epub ahead of print).

14. Noble C, Abbas A, Lees C, **Ho G.T.**, et al. The Characterisation of Distinct Intestinal Gene Expression Profiles in Ulcerative Colitis by Microarray Analysis. Gastroenterology 2007 (*submitted*).

15. Lees CW, Noble C, Tremelling M, Nimmo ER, Cornelius J, Tenesa A, **Ho G.T.**, et al. The hedgehog signalling pathway is dysregulated in ulcerative colitis. Nature Genetics 2007 (*submitted*).

16. Arnott ID, **Ho G.T.**, Nimmo ER, Satsangi J. Toll-like receptor 4 gene in IBD: further evidence for genetic heterogeneity in Europe. Gut 2005 Feb; 54(2):308

17. Lees CW, Heys D, **Ho G.T.**, et al. Infliximab in acute severe ulcerative colitis: Saving colons or risking lives? Alimentary Pharmacology and Therapeutics 2007 (accepted manuscript).

16. **Ho G.T.**, Mowat C, Satsangi J. Predicting outcome of ulcerative colitis: The use of clinical and genetic predictive factors. Inflammatory Bowel Disease Monitor 2002; 11:200-209.

Abstracts

1. **Ho G.T.**, Noble C, Cornelius J, Penman ID, Arnott IDR, Diehl L and Satsangi J. The Human ATP-binding cassette (ABC) transporters and xenobiotic-transcription regulators: Analysis of epithelial gene expression in inflammatory bowel disease. Gastroenterology 2007 (A341) (oral presentation in Research Forum, Digestive Diseases Week 2007) and Gut (A347) (Best Poster in IBD section – presented at the plenary session British Society of Gastroenterology 2007).

2. **Ho G.T.**, Soranzo N., Tenesa A., Nimmo E.R., Drummond H.E., Goldstein D.B. and Satsangi J. Gene-wide haplotypic analysis demonstrates association between ATP-Binding cassette 3/ Multi Resistance Protein 3 (ABCC3/MRP3) gene and inflammatory bowel disease. Gastroenterology 2006: (Poster of Distinction A518) (Plenary poster BSG 2007).

3. **Ho G.T.**, Smith L, Aitken S, Rose J, Ruddell W, Bramley P, Arnott ID, Shand AG, Palmer KR, Satsangi J. The use of adalimumab in refractory Crohn's disease: A tertiary referral centre experience. Gut A001; 2007 (oral presentation British Society of Gastroenterology 2007).

4. **Ho G.T.**, Soranzo N, Nimmo ER, Tenesa A, Goldstein DB, Satsangi J. Specific haplotype variations of the ABCB1/MDR1 gene can confer risk to, or protection from ulcerative colitis. Presented at the Medical Research Society (MRS) meeting, London (February 2005) and Gut A123 (Plenary Poster BSG 2005).

5. **Ho G.T.**, Chiam PS, Drummond H, Loane J, Arnott ID, Satsangi J. The efficacy of corticosteroid therapy: analysis of a 5-year UK inception cohort. Gastroenterology A456; 2005 (Digestive Diseases Week).

6. **Ho G.T.**, Nimmo ER, Drummond HE, Fennell J, Arnott ID, Mowat C, Satsangi J. Association of multidrug resistance gene (MDR1) gene with extensive and severe ulcerative colitis. Oral presentations at the BSG 2004 and DDW 2004

7. **Ho G.T.**, Seddon AJ, Therapondos G, Satsangi J, Hayes PC. The clinical course of ulcerative colitis before and after liver transplantation for primary sclerosing cholangitis. Oral presentation at the BSG 2004 (Glasgow)

8. **Ho G.T.**, Mowat C, Fennell J, Shah N, Drummond H, Prescott RJ, Satsangi J. Predicting outcome in severe ulcerative colitis: Development of a novel predictive risk model to aid early selection of patients for second-line medical therapy or surgery. Oral presentation at the DDW 2003 (Atlanta)

9. Din S, **Ho G.T.**, Hogarth C, Lennon AM, Arnott ID, Satsangi J.et al. Proteomic profiling identifies corticosteroid resistance in severe ulcerative colitis. Poster of distinction, DDW 2004 (Chicago)

10. **Ho G.T.**, Gunn RJ, Torkvist L, Loane J, Lessells AJ, Malcomson R, Satsangi J. Does histological and clinical activity predict risk of colorectal cancer in ulcerative colitis? Poster of distinction, DDW 2003 (New Orleans).

Book Chapters:

1. **Ho G.T.**, Lees CW, Satsangi J. Ulcerative Colitis. Medicine International, Elsevier Ltd. May 2007 (DOI 10.1016/j.mpmed.2007.02.002)

2. Lees CW, **Ho G.T.**, Satsangi J. Genetics of inflammatory bowel disease. Encyclopaedia of Life Sciences, Wikipaedia Ltd., 2007 (Accepted manuscript).

Chapter 1

Introduction

1. Introduction

Ulcerative colitis represents one of the entities of inflammatory bowel diseases, a global chronic idiopathic inflammatory condition affecting the colon and rectum. Increasing data provide evidence of a complex interplay between components of the innate immune system and environmental factors, notably the microflora of the intestinal mucosa. A widely accepted hypothesis is that ubiquitous, commensal intestinal bacteria trigger an inappropriate, overactive, and ongoing mucosal immune response that mediates intestinal tissue damage in genetically susceptible individuals (Sartor 2006). There are now also many studies suggesting that defective primary intestinal barrier defence plays an important role in the pathogenesis of IBD and more recently has been referred to as 'an archetypal barrier disease'(Schreiber et al. 2005).

1.1 Clinical features of ulcerative colitis

1.1.1 Epidemiology

In the United Kingdom, the incidence of UC is approximately 10/100 000 with a point prevalence of 200/100 000. Ulcerative colitis may present at any age but typically in the 3rd to 4th decade (later than Crohn's disease). Men and women are equally affected. The incidence rates for UC have remained constant in areas such as Northern Europe and North America. In areas with previously low incidence, such as Southern Europe and Asia, the incidence appears to be increasing although differences in case ascertainment and study design introduce a degree of difficulty in comparing these studies(Loftus, Jr. 2004). In adults at presentation about 55% have proctitis, 30% left sided colitis, and 15%

extensive colitis or total colitis. In children, only 25% present with proctitis alone, 30% have left sided colitis, and in 45% the disease extends to the transverse colon or beyond.

1.1.2 Clinical presentation

The landmark paper by Felicity Edwards and Sidney Truelove in 1963, described the clinical pattern and prognosis of 624 patients between 1938 and 1962. The age of onset was <20 years in 10.0%, 20-59 years in 72.8% and >60 years in 17.2%. The median decade of onset was 30-39 years (26.9%)(Edwards F.C. and Truelove 1963). This historical data is in agreement with more recent studies(Riegler et al. 2000; Lennard-Jones and Shivananda 1997). The major symptoms of UC include diarrhoea, rectal bleeding and colicky abdominal pain. In a prospective Europe-wide study involving 1379 patients with UC presented over a two-year period, the predominant presenting symptoms were increased bowel frequency (93%) and visible blood in stools (96%)(Lennard-Jones and Shivananda 1997). This is in contrast with Crohn's disease where weight loss is predominant (73%) and visible blood in stools occurring in only 48% of patients. The clinical presentation varies widely but is determined by two important factors – disease extent and severity. In patients with limited disease (rectosigmoid involvement), the symptoms of rectal irritation namely tenesmus (the sensation of incomplete emptying), small volume diarrhoea, proximal constipation and rectal bleeding predominate. In contrast, in extensive colitis (beyond the splenic flexure), profuse bloody diarrhoea, abdominal cramping and systemic features such as weight loss, fever and tachycardia are more prominent. Symptoms tend to present insidiously but may also present acute mimicking infective an infective aetiology.

1.1.3 Diagnosis

The diagnosis of UC is principally based on clinical, endoscopic and histological grounds. UC invariably affects the rectum and extends proximally to a variable distance. The inflammation is characteristically confluent, with increased granularity and haemorrhagic appearances. The earliest signs tend to be subtle loss of vascular patterns with hyperaemia and oedema of the mucosa. With more active inflammation, the mucosa becomes granular with presence of mucopus and contact bleeding. In advanced cases, deep ulcerations may present which may mimic severe Crohn's disease. In diagnosis of UC, 2 critical features are influential in the subsequent management namely: 1) disease extent and 2) disease severity.

Assessment of disease extent

Patients with extensive disease (macroscopic evidence of colitis beyond the splenic flexure) have an increased likelihood of medically refractory and severe disease. A review of selected referral centre-based (Ritchie, Powell-Tuck, and Lennard-Jones 1978; Moum et al. 1997) and population based cohorts (Farmer, Easley, and Rankin 1993; Langholz et al. 1994) have shown that the actuarial risk of colectomy is influenced by disease extent. The risk of colectomy among patients with only limited proctitis ranged from 2-9% while patients with extensive colitis had five-year colectomy rates of 30 to 44 %. The extent of disease in the acute setting can often be determined with non-invasive methods such as a plain abdominal X-ray (demonstrating empty and/or oedematous colon in the affected regions). Some centres advocate the use of colonoscopy but often in the

acute setting, the medical treatment is unchanged regardless of disease extent, therefore such practice is avoided for fear the precipitating a complication. In the subacute or quiescent phase, disease extent can often be determined using histological methods if the colonoscopic evidence is equivocal. Disease extent is not a static phenomenon and the risk of disease extension of distal disease is a subject of debate. In a retrospective study of 145 patients with distal colitis at presentation, disease extension proximal to sigmoid was 36% at a median of 6 years, becoming extensive in 29%. Using actuarial analysis, disease extension was predicted for 16% (95% CI 11-24%) at 5 years and 31% (95% CI 23-40%) 10 years after diagnosis(Ayres et al. 1996). In a further study, Langholz et al. in a longitudinal population-based actuarial analysis suggest that 30% of patients with originally limited disease may progress to more extensive disease after 5 years of follow-up(Langholz et al. 1994). If progression can occur in limited disease, then regression might be expected in a proportion with extensive disease. In a cohort comprising of 399 patients with UC, endoscopic regression occurred in 22% with histological regression in 24%(Moum et al. 1997). It is of course noteworthy that patient with extensive disease may have no clinical or histological evidence of colitis when in remission (indeed in this study, 30% of patients had a subsequently normal colonoscopy 14 months after diagnosis). Patients with extensive disease have an increased long term risk of colorectal cancer/dysplasia.

Assessment of disease severity

The risk of colectomy in UC is also strongly associated with initial presentation with severe attack of UC(Sinclair, Brunt, and Mowat 1983; Langholz et al. 1994). The severity of disease is defined clinically by a series of observations basically describing profuse bloody diarrhoea with the presence of systemic sypmtoms. The most widely use criteria was defined by Truelove and Witts by the presence of stool frequency (>6 bloody motions/day) together with one or more of these features, tachycardia, fever, anaemia or elevated inflammatory markers (ESR > 30mm/hour). In the landmark paper describing the first controlled trial of oral cortisone in patients with ulcerative colitis in 1955, the mortality rate from severe UC in the placebo arm was 24%(Truelove SC and Witts LJ 1955). The morbidity associated with severe attacks of UC remains high and much of this is associated with delayed surgery and also prolonged exposure to high-dose corticosteroid therapy. It remains uncertain however, which patients will develop severe disease and in those who have severe disease, which patients are unlikely to respond to corticosteroids. In a long term follow-up study involving 1116 patients in the Cleveland Clinic diagnosed with UC between 1960 and 1983, Farmer et al. demonstrated that 13% of these patients presented with severe UC within 2 years of diagnosis(Farmer, Easley, and Rankin 1993). It is noteworthy that Langholz et al., in a cohort of 1161 patients with UC based in Copenhagen, Denmark, also showed that 9% of patients required colectomy for severe disease within the first 2 years of presentation(Langholz et al. 1994).

1.1.4 Current therapeutic strategies and problems

Induction of remission

As with the clinical course of UC, treatment strategies depend on 2 critical factors: namely disease extent and severity. The focus of treatment is based on the induction and maintenance of remission. 5-aminosalicylic acid (sulphasalazine and mesalazine) is considered as first-line therapy for active mild-moderate, left-sided or extensive ulcerative colitis. With modern delayed-release formulations, conventional doses are very well tolerated, and there is accumulating evidence that increasing the dose, to >4 g/day, leads to higher response rates and earlier symptom relief in patients with ulcerative colitis(Sutherland and Macdonald 2006a; Hanauer 2006). The combination of oral and rectal (enema) formulations of 5-aminosalicylic acid leads to faster and higher remission rates(Vecchi et al. 2001; Marteau et al. 2005). Despite the fact that clinical trials in ulcerative colitis have often used different endpoints, making it difficult to make objective comparisons, the evidence now strongly suggests that it is appropriate to start patients with mild or moderately active ulcerative colitis on doses of 5-aminosalicylic acid of at least 4 g/day(Hanauer 2006; Travis 2006). In left-sided disease, the treatment of choice is topical therapy namely by the use of foam or retention enemas, and suppositories. The use of topical therapy is advantageous in delivering high doses of anti-inflammatory therapy directly to the site of inflammation with little systemic absorption and adverse effects. Typically the choice of topical therapy is either 5-aminosalicylate or corticosteroids enemas. Recent data have shown that 5-ASA is more efficacious than the latter, and therefore should be used as first-line. In patients with proctitis (rectal involvement only), the use of enemas can by-pass the rectum and therefore result in

apparent lack of efficacy. In these circumstances, rectal 5-ASA suppositories may be more useful. The use of topical therapy is often the subject of personal preference; but is superior to oral 5-ASA in distal disease. Traditionally, oral 5-ASA is given in the form of sulphasalazine (5-ASA as an active ingredient linked to a carrier sulphpyridine molecule which is absorbed systemically after the digestion of the Azo-bond by bacteria). The more modern 5-ASA therapy is based on mesalazine formulation which relies on either pH-dependent or time-release release formulation in the colon. Although the Cochrane Systematic review revealed no differences in efficacy and cost-effectiveness between sulphasalazine and mesalazine, the use of 5-ASA has shifted away from the sulphasalazines due to the adverse effect of profiles of headache, nausea and in males, reversible oligospermia(Sutherland and Macdonald 2006b; Sutherland et al. 2002).

In patients with more extensive or severe disease, or with disease unresponsive to oral 5-ASA, corticosteroids are required to induce remission. Although effective in the short term (in up to 70% of the cases), the adverse effect profile of corticosteroids needs to be borne in mind as a substantial proportion of patients will relapse or fail to respond in the medium term(Truelove SC and Witts LJ 1955). Based on 2 inception cohort studies, approximately 50% of patients treated with corticosteroids will either require surgery or be corticosteroid dependent after one year of the initiation of therapy(Faubion et al. 2001; Ho et al. 2006a). Patients with extensive and severe disease are more likely to fail to respond to corticosteroids(Ho et al. 2006a; Jarnerot, Rolny, and Sandberg-Gertzen 1985). The use of corticosteroids indeed has no place in the maintenance of remission of UC. Although factors such as underdosing and abrupt tapering of corticosteroids could be

responsible for failure of this therapy, a prospective study involving 48 consecutive patients with active CD, using a higher oral corticosteroids dose and a slower tapering schedule than used in the previous studies reported steroid dependency and resistance rates of 63% and 13%, respectively(Reinisch et al. 1995). In patients with acute severe UC requiring intensive in-patient medical therapy, the typical dosing regime would be 60mg/day of intravenous methylprednisolone or 400 mg of hydrocortisone, for 5 days. Oral corticosteroids are usually 40-60mg/day and reducing by 5mg/week (total duration of therapy – 2 months)(Carter, Lobo, and Travis 2004).

There is considerable data on the short- and long-term use (with safety data) of ciclosporin in acute severe UC. Ciclosporin acts mainly by inhibiting T lymphocyte function, which is essential for the propagation of inflammation. Unlike most other immunosuppressive agents, ciclosporin does not suppress the activity of other haematopoietic cells, does not cause bone marrow suppression and has a rapid onset of action. Controlled trial data have shown that high-dose intravenous ciclosporin (4 mg/kg) induces clinical response in 60–80% of patients with severe active ulcerative colitis(Lichtiger et al. 1994; Carbonnel et al. 1996). Although most side-effects are minor and managed with mild adjustments in dose, the incidence of severe and sometimes fatal opportunistic infections, most notably *Pneumocystis carinii* pneumonia, raises serious concerns and has to an extent restrict the usefulness of such treatment(Quan et al. 1997). Results data have shown that a lower dose of intravenous ciclosporin (2 mg/kg) or ciclosporin monotherapy are equally in severe ulcerative colitis without increased toxicity(Van Assche et al. 2003; D'Haens et al. 2001). Long-term follow-up studies of

patients with severe ulcerative colitis treated with ciclosporin have shown that 53–62% avoid colectomy at 3–5 year follow-up(Stack, Long, and Hawkey 1998; Cohen, Stein, and Hanauer 1999; Campbell, Travis, and Jewell 2005). Nevertheless, the use of ciclosporin is based on the premise of bridging to more effective longer term immunosuppression. Addition of azathioprine or 6-mercaptopurine to treatment regimens is recommended for all patients who respond to ciclosporin, since these drugs help avoid further colectomy in the transition to long-term therapy and might give a better quality of life than colectomy(Cohen RD, Brodsky AL, and Hanauer 1999). The use of oral ciclosporin is considerably less efficacious, but results from unmasked studies suggest that microemulsion ciclosporin (Neoral), which has better oral absorption, could be a more cost effective maintenance therapy for some patients(Actis et al. 1998; Navazo et al. 2001).

The use of infliximab, a chimeric monoclonal antibody directed against tumour necrosis factor-alpha (TNF-α) has recently been shown to be effective in moderate to severe UC. In the ACT-1 and 2 randomized controlled trial involving a total of 728 patients comparing the use of infliximab with placebo, significantly higher response rates where observed after 8 weeks and 1 year of therapy(Rutgeerts et al. 2005). In ACT 1 at week 8, 69.4 percent of patients in the group receiving 5 mg/kg of infliximab (84 of 121) and 61.5 percent of patients in the group receiving 10 mg/kg of infliximab (75 of 122) had had a clinical response, as compared with 37.2 percent of patients in the placebo group (45 of 121, $P<0.001$ for both comparisons). In ACT 2 at week 8, 64.5 percent of patients in the group receiving 5 mg of infliximab (78 of 121) and 69.2 percent of patients in the group

receiving 10 mg of infliximab (83 of 120) had had a clinical response, as compared with 29.3 percent of patients in the placebo group (36 of 123, P<0.001 for both comparisons). In both studies, the proportions of patients who had a clinical response or remission at weeks 8 and 30, and at week 54 in the ACT 1 trial, were higher by a factor of 1.7 to more than 2 in the infliximab groups than in the placebo groups. The rates of clinical response were similar between the subpopulations of patients who were corticosteroid-refractory and those who were not corticosteroid-refractory. In an earlier controlled-trial, infliximab was not found to be superior in the subgroup of patients with moderate to severe corticosteroid dependent/resistant UC(Probert et al. 2003). In acute moderate to severe UC, Jarnerot et al. has demonstrated that a single infusion of infliximab at 5mg/kg to be effective in inducing remission (p=0.017, odds ratio 4.7)(Jarnerot et al. 2005a). Patients were randomized to infliximab/placebo either on day 4 after the initiation of corticosteroid treatment if they fulfilled the index criteria for fulminant ulcerative colitis on day 3 or on day 6–8 if they fulfilled index criteria on day 5–7 for a severe or moderately severe acute attack of ulcerative colitis with the primary end-points being colectomy or death 3 months after initiation of therapy. There were no differences in adverse effects in this study. There is no extensive experience in the use of infliximab in UC and the long term safety and efficacy of infliximab in UC is unclear. The current strategy and positioning of infliximab, as with ciclosporin, is to use this as a 'bridge' to longer term immunosuppressive maintenance treatment such as azathioprine.

Maintenance of remission

The cornerstone of maintenance of remission is based on 5-ASA therapy. Several clinical studies have shown that the use of 5-ASA therapy reduces the likelihood of relapse over a year-therapy by threefold (from a likelihood of relapse of 70-80% without therapy to 10-30% on 5-ASA maintenance therapy per year). Systematic reviews have confirmed the superiority of sulphasalazine and mesalazine in maintaining remission(Sutherland and Macdonald 2006a). A Peto odds ratio of 0.47 (95% CI, 0.36 to 0.62) and number-needed to treat of 6 based on meta-analysis of 881 patients with the failure to maintain endoscopic or clinical remission used as outcomes (withdrawals and relapses as defined by each study). 5-ASA was observed to be significantly more effective than placebo in all dosage subgroups (<1 g/d, 1-1.9 g/d, >or=2 g/d); however, a dose-dependent trend was not observed (P = 0.489). There are less data regarding dose-related benefits of aminosalicylates to maintain remissions in UC greater than 1.6 g/day of mesalazine, although the absence of dose-related side effects allows continuation of the same inductive dose through maintenance treatment without dose-related toxicity(Hanauer et al. 2005; Paoluzi et al. 2005; Fockens et al. 1995).

Azathioprine and 6-mercaptopurine are purine analogues that competitively inhibit the biosynthesis of purine nucleotides. Azathioprine is the pro-drug of 6-mercaptopurine. These immunosuppressants are established therapy in the management of IBD and have been used in the clinical setting for 30 years with clinical benefit evident in up to two-thirds of the patients although the specific mode of action in IBD is not well-understood (George et al. 1996; Pearson DC et al. 1995; George et al. 1996; Lamers CB et al. 1999).

Clinical data pertaining to their respective efficacy are based on older clinical studies (Jewell and Truelove 1974; Kirk and Lennard-Jones 1982; Rosenberg et al. 1975; Hawthorne et al. 1992; Fraser, Orchard, and Jewell 2002). The use of Azathioprine has been focussed on the group of patients with corticosteroid-dependent UC or frequent relapses (>2 episodes/year). In a recent study by Ardizzone et al., azathioprine has been shown to be particular benefit in patients with corticosteroid dependency compared with mesalazine therapy(Ardizzone et al. 2006). In this study, significantly more patients in the azathioprine than in the 5-aminosalicylic acid group had clinical and endoscopic remission, and discontinued steroid therapy, both in the intention to treat (azathioprine v 5-aminosalicylic acid: 19/36 patients (53%) v 7/36 (21%); odds ratio (OR) 4.78 (95% confidence interval (CI) 1.57-14.5)) and per protocol (azathioprine v 5-aminosalicylic acid: 19/33 patients (58%) v 7/34 (21%); OR 5.26 (95% CI 1.59-18.1)) analysis(Ardizzone et al. 2006). One limitation of this drug is that adverse drug reactions to AZA or 6-MP occur in 15% to 28% of patients(Present et al. 1989; Schwab et al. 2002; Lennard 2002; Ansari et al. 2002; Sandborn 1998) and often necessitate withdrawal of therapy. These side-effects are typically gastrointestinal in nature (nausea, vomiting and abdominal pain – up to 28%) and less commonly leucopaenia (5%), pancreatitis (3%), hepatitis (0.3%) and infections (7.4%). The most serious adverse effect is profound bone marrow suppression in patients (1 in 300 individuals) who are deficient in thiopurine methyl transferase (TPMT) enzyme involved in the metabolism of Azathioprine/6-mercaptopurine. Genetic polymorphisms within the TPMT gene are associated with this deficiency and the genotype or TPMT activity is routinely measure at or before the commencement of this therapy(Black et al. 1998; Collie-Duguid et al. 1999; Colombel et

al. 2000; Evans et al. 1991). In patients who cannot tolerate Azathioprine due to predominantly gastrointestinal side-effects (excluding those with hypersensitive or idiosyncratic reactions – pancreatitis and severe myelosuppression), 6-mercaptopurine can be used and effective in up to 50% in this subgroup(Carter, Lobo, and Travis 2004). The therapeutic regime is based on 2mg/kg for Azathioprine or 1 mg/kg for 6-mercaptopurine. The therapeutic effect for Azathioprine and 6-mercaptopurine is usually optimally observed 2-3 months after the initiation of therapy therefore a good overlap with corticosteroid therapy is often necessary. Patients who take azathioprine should avoid allopurinol (a xanthine oxidase inhibitor) as it inhibits the breakdown of azathioprine and results in an increased risk of myelosuppression.

Other therapies such as methotrexate(Oren et al. 1996; Cummings et al. 2005), tacrolimus(Baumgart et al. 2006; Hogenauer et al. 2003), mycophenolate mofetil(Orth et al. 2000), heparin(Ang et al. 2000; Panes et al. 2000; Dotan I et al. 2001; Torkvist L et al. 1999), nicotine, antibiotics(Gionchetti et al. 2006), probiotics(Bibiloni et al. 2005), biological therapies such as anti-CD25 (basiliximab) (Creed et al. 2003; Van Assche et al. 2006), anti-alpha4 beta7 integrin (natalizumab)(Feagan et al. 2005), ICAM-1 (alicaforsen)(van Deventer et al. 2006), epidermal growth factor enemas(Sinha et al. 2003); helminth therapy(Summers et al. 2005), leukocyte apheresis(Sandborn 2006) and aloe vera(Langmead et al. 2004) have been described. These therapies are not presently established within clinical practice, in particular some biological therapies remain in evaluation within clinical trials.

1.1.5 Corticosteroid resistance/dependence in IBD

As discussed earlier, corticosteroid therapy remains the first-line therapy in the treatment of active moderate to severe IBD. It is evident that a subset of patients will fail to respond to this treatment, whether acutely or chronically and require surgery as a consequence. It is conceivable that the both elements of the function of 'aggressive' disease and corticosteroid resistance play significant roles in the ultimate failure of medical therapy and the need for surgery as the definitive treatment for UC. It is often assumed that responsiveness to corticosteroid treatment in IBD is simply a function of disease severity. However, even in some mild cases, failure of this therapy is not uncommonly seen. As with other inflammatory conditions, this phenomenon is also seen in conditions such as rheumatoid arthritis, asthma and systemic lupus erythematosus (SLE)(Carmichael et al. 1981; Seki M et al. 1998; van Schaardenburg D et al. 1995).

Historically, two pivotal studies have highlighted the scale of the problem associated with corticosteroid resistance and dependence(Munkholm et al. 1994; Faubion et al. 2001). Munkholm and colleagues prospectively studied the outcome of the first corticosteroid treatment in patients with newly diagnosed Crohn's disease between 1979 and 1987 in Copenhagen, Denmark(Munkholm et al. 1994). In this cohort, 109 patients (56%) were treated with corticosteroids. Complete remission was obtained in 48%, partial remission in 32%, and no response in 20% within 30 days of treatment. Among the primary responders (complete and partial remission), only 55% remained in prolonged response after treatment had finished, while as many as 45% relapsed or could not be withdrawn from treatment within one year. There were no relationships between the outcomes of

therapy with disease localization, age, sex or clinical symptoms. Therefore, a prolonged steroid response was only evident in 44%; with 36% and 20% of patients demonstrating corticosteroid dependence and resistance respectively.

Faubion and colleagues further demonstrated that the rates of corticosteroid dependence and resistance were similar in Crohn's disease and UC(Faubion et al. 2001). In this inception cohort based in Olmstead County, Minnesota, all newly diagnosed patients (173 Crohn's disease and 185 UC) diagnosed between 1970 and 1993 were assessed. The investigators found the use of corticosteroids at diagnosis to be common, seen in 43% and 34% patients with UC and Crohn's disease respectively. Within 30 days of the initiation of therapy, 58% and 26% of patients with Crohn's disease responded completely and partially respectively; whereas 16% showed no response. In UC, 54% and 30% responded completely and partially respectively; whereas 16% showed no response. One-year outcomes for Crohn's disease were prolonged response in 24 (32%), corticosteroid dependence in 21 (28%), operation in 28 (38%), and lost to follow-up in 1 (1%). One-year outcomes for ulcerative colitis were prolonged response in 31 (49%), corticosteroid dependence in 14 (22%), and operation in 18 (29%). A high frequency of surgical intervention was reported in corticosteroid dependent (26%) and resistant patients (59%) within 1 month after corticosteroid treatment(Munkholm et al. 1994). Although factors such as underdosing and abrupt tapering of corticosteroids could be responsible for failure of this therapy, a prospective study involving 48 consecutive patients with active CD , using a higher oral corticosteroids dose and a slower tapering

schedule than used in the previous studies reported steroid dependency and resistance rates of 63% and 13%, respectively(Reinisch et al. 1995).

1.1.6 Pharmacogenetics in corticosteroid therapy

In general, although the molecular mechanisms of corticosteroids are well-known, the underlying causes accounting for the highly variable efficacy of corticosteroid resistance and dependence remains unresolved in inflammatory bowel disease and other inflammatory conditions such as asthma and rheumatoid arthritis(Payne and Adcock 2001). In IBD, the overexpression of P-glycoprotein 170 (PgP, an efflux transporter gene encoded by the multidrug resistance gene, MDR1/ABCB1) has been suggested to play an important role. Compelling data from Farrell et al. showed that PgP expression on peripheral blood lymphocytes in patients with IBD who have required surgery as a consequence to failure of corticosteroid therapy, were increased compared to those patients who have required surgery for dysplasia/obstruction(Farrell et al. 2000). Recent genetic data have demonstrated an association between carriage of an exonic variant in the gene, single nucleotide polymorphism (SNP) C3435T with the expression levels of PgP(Hoffmeyer et al. 2000a). These findings are clearly exciting in light of the possibility of identifying patients who are less likely to respond to corticosteroids using a simple genetic test. More recently, the C3435T polymorphism has been shown to be associated with drug-resistant epilepsy(Siddiqui et al. 2003). Patients with drug-resistant epilepsy were more likely to have the CC genotype (high PgP expression phenotype) compared with the TT genotype (odds ratio 2.66, 95% CI 1.32-5.38, p=0.006). Fellay et al. demonstrated that this SNP also predicts immune recovery after initiation of antiretroviral

treatment in HIV-infection. Patients with the TT genotype 6 months after starting treatment had a greater rise in CD4-cell count than patients with the CT and CC genotype ($p=0.0048$), and the best recovery of naive CD4-cell(Fellay et al. 2002). The pharmacogenetic contributions of these SNPs in predicting surgery in IBD have been studied and will be discussed in Chapter 4.

Mutations of the glucocorticoid receptor gene (GR) associated with functional changes in the glucocorticoid receptor resulting in clinical corticosteroid resistance syndromes have been described, but these are rare(DeRijk RH et al. 2002). However, splice variants of the human glucocorticoid receptor are commonly present (GR-A, GR-B, GRβ, GRγ and GR-P). The GRβ (an isoform of the GR-gene) in particular, has been shown to be associated with glucocorticoid resistance although its particular function remains unclear. Honda and colleagues reported that, GRβ mRNA was detected in 83% of patients with corticosteroid-resistant ulcerative colitis but present only in 10% of glucocorticoid sensitive patients and controls(Honda M et al. 2000). Increased GRβ expression has been associated with corticosteroid resistance in several other inflammatory conditions, most consistently in asthma(Hamid Q et al. 1999; Hamilos DL et al. 2001; Leung DY et al. 2000). Additional data suggests that the phenomenon of corticosteroid resistance is not generalized but somehow compartmentalized to T-lymphocytes and possibly other target inflammatory cells. In a prospective study of 18 patients with acute severe UC, T-lymphocyte corticosteroid resistance was found to be an important factor in determining response to corticosteroid treatment(Hearing et al. 1999). The clinical relevance of these findings have now led to the development of a novel therapy, basiliximab (anti-CD25)

which acts as a corticosteroid-sensitizing agent and is currently under evaluation(Creed et al. 2003).

In asthma, a reduced inhibitory effect of corticosteroids on Activated Protein-1 (AP-1) activation and cytokine expression has been shown in corticosteroid resistant patients, possibly due to enhanced AP-1 activation(Matthews et al. 2004; Adcock et al. 1995) (Sousa et al. 1999). The resultant increased activation of AP-1 was postulated to lead to sequestration of GR, thus preventing interaction with other proteins resulting in resistance at the site of inflammation where cytokines are produced, for example, in the airways of asthmatic patients but not at non-inflamed sites. This may explain why patients with corticosteroid-resistant asthma are not resistant to the endocrine and metabolic effects of corticosteroids and therefore develop corticosteroid side-effects(Lane et al. 1996). Other explanations for these differential responses have also been suggested including abnormal histone deacytelation activity in controlling gene transcription(Ito et al. 2006).

1.1.7 Surgery

The indications for surgery are usually categorised into emergency and elective operations. In the former, the absolute indications for surgery are perforation, massive haemorrhage with commoner indications being failed medical therapy and toxic megacolon. The timing of emergency in the case of failed medical therapy or toxic megacolon is dependent on careful joint medical-surgical decision. The consideration of whether second-line medical therapy is indicated ahead of early colectomy in severe disease can be difficult. Some centres view the presence of megacolon as an indication

itself for surgery whereas other centres adopt a more conservative medical approach(Kamm 2004; Cima and Pemberton 2004). For the elective indications for surgery, these are usually based on patients with chronic continuous symptoms, corticosteroid dependency, partial response to medical therapy and the presence of malignancy/dysplasia. Generally, 4 surgical approaches are considered:

- Total colectomy with end ileostomy
- Proctocolectomy with the formation of ileo-anal pouch (IPAA).
- Protocolectomy with ileo-rectal anastamosis
- Proctocolectomy with the formation of a continent ileostomy or Kock's pouch.

There is general agreement that the operation of choice in the emergency situation is colectomy with ileostomy and the preservation of the rectum(Nicholls 2002). In the acute post-operative setting, the risk of serious complication relates in part to the possibility of rectal stump dehiscence. In this setting, the principle of operation has centred on the preservation of a lengthy rectal remnant (with or without a mucous fistula) which would enable the exteriorization in the case of rectal stump leak or retraction.

The choice of surgery is rather individualised in part depending on patient's perceptions and psycho-social situations. Total proctocolectomy with construction of an ileal pouch anal anastomosis (IPAA) has become the standard of care for patients with UC who require removal of the colorectum. This surgery involves abdominal colectomy and construction of an ileal pouch that is anastomosed to the anus. In younger patients, the option of ileo-anal pouch is more attractive as it would avoid the need of a stoma thus preserving continence allowing a more active lifestyle e.g. sports. The majority of patients with UC that undergo IPAA report improved quality of life after surgery (Bach

and Mortensen 2006). A growing number of complications however, can impact adversely on the long-term outcome of these patients. The most common and widely reported of these complications is a nonspecific inflammation of the ileal pouch, a condition referred to as "pouchitis". Pouchitis is manifest clinically as a syndrome of watery, frequent diarrhea or hematochezia, accompanied by urgency, incontinence, abdominal cramping, malaise, and fever. The cumulative probability of pouchitis, determined on the basis of symptoms, endoscopy, and histopathology in 468 IPAA patients was 20% at 1 year, 32% at 5 years, and 40% at 10 years(Lepisto, Luukkonen, and Jarvinen 2002). In contrast, no pouchitis occurred following operation for FAP (7% of the total). These rates are in accordance with those of other centers. The incidence of pouchitis appears to be independent of surgical technique with respect to pouch construction, use of a defunctioning stoma, or laparoscopic techniques (Oresland et al. 1990; Heuschen et al. 2001a; Sagar et al. 1992; Pace et al. 2002). Patients with primary sclerosing cholangitis are more prone to develop pouchitis, with a cumulative probability of 79% at 10 years(Penna et al. 1996). Persistence of extraintestinal manifestations of UC has also been linked to an increased risk of developing pouchitis, and certain patients exhibit a temporal relationship between their pouchitis and extraintestinal symptoms akin to that described for UC, fueling speculation that these 2 inflammatory processes represent variations of the same underlying condition(Lohmuller et al. 1990). Perpetuating this theme, smoking is considered to be protective against UC(Beaugerie et al. 2001) and also reduces the incidence of pouchitis(Merrett et al. 1996; Stahlberg et al. 1996). The incidence of pouch failure is reported to be 10% at 10 years, with severe pouchitis accounting for 10% of failures(Bach and Mortensen 2006).

Other complications of IPAA include the subsequent development of Crohn's disease in the pouch, cuffitis (inflammation in the rectal cuff), and irritable pouch syndrome (symptoms of abdominal pain and/or diarrhea in the absence of ileal pouch mucosal inflammation).In some cases, ileo-rectal anastamosis maybe a more attractive option in young females patients in order to avoid protectomy and pelvic dissection, with the issues of fecundity and fertility in mind. Johnson et al examined fertility rates in married or cohabiting females ages 18 to 44 years with a history of UC(Johnson et al. 2004). Of 153 patients, 59 (38%) who had undergone IPAA were unable to conceive following 1 year of unprotected intercourse compared with 8/60 patients (13%) subject to medical management alone. The authors concluded that whereas a diagnosis of UC did not affect fertility, pouch surgery was associated with a 98% reduction. Surgeons may consider delaying proctectomy until a family has been established.

1.1.8 Malignancy risk

Patients with ulcerative colitis have a higher risk of colorectal cancer than the general population. Disease extent and duration are the two critical factors underlying this association although recent data also suggest disease severity may also be influential. Ekbom and colleagues reported standardised incidence ratios for colorectal cancer risk of 1.7 for patients with proctitis, 2.8 for patients with disease extending beyond the rectum but no further than the hepatic flexure, and 14.8 for patients with disease extending beyond the hepatic flexure(Ekbom et al. 1990). A family history of colorectal cancer(Askling et al. 2001)and the presence of primary sclerosing cholangitis(Jayaram, Satsangi, and Chapman 2001) and backwash ileitis(Heuschen et al. 2001b) are also independently associated with increased cancer risk. Continuation of maintenance 5-aminosalicylate(Eaden et al. 2000) and sulfasalazine(Pinczowski et al. 1994), cigarette smoking(Pinczowski et al. 1994), and possibly folic-acid[1] and vitamin-E supplementation are protective factors(Lashner et al. 1997). In a study by Eaden et al., a relative reduction of 75% in risk of cancer was demonstrated in those patients regularly taking 5-aminosalicylates, even adjustment for low-dose mesalazine (1.2 g daily)(Eaden et al. 2000). A further study by Rutter et al., showed that histological evidence of severe disease further substratify patients who developed dysplasia or malignancy(Rutter et al. 2004a). The biological explanation of the protective effect of 5-ASA is unclear but may represent a surrogate marker of good disease control. Colorectal cancer in ulcerative colitis is more likely to have a uniform distribution throughout the colon, affect more than one area, and present at a higher grade at diagnosis(Ekbom et al. 1990). Recognition of dysplasia as a precursor to cancer in ulcerative colitis supports the rationale for

performing regular surveillance by colonoscopy, but the benefits, reliability, and cost of surveillance programmes are not clear(Eaden and Mayberry 2002; Rutter et al. 2006). The current guidelines recommend those patients who have had extensive ulcerative colitis for 7–8 years and those who have had left-sided disease for 15 years should be enrolled in surveillance programmes, but there are inconsistencies exist in clinical practice. A further recent study by Rutter et al., suggest that endoscopic appearances predict the risk of dysplasia/malignancy(Rutter et al. 2004b). In this case-control study, cases were significantly more likely to have post-inflammatory polyps (OR 2.14 95% confidence interval 1.24-3.70), strictures (OR 4.22; 1.08-15.54), shortened colons (OR 10.0; 1.17-85.6), tubular colons (OR 2.03; 1.00-4.08), or segments of severe inflammation (OR 3.38; 1.41-10.13), and less likely to have had a macroscopically normal looking colonoscopy (OR 0.40; 0.21-0.74). After multivariate analysis, a macroscopically normal looking colonoscopy (OR 0.38; 0.19-0.73), post-inflammatory polyps (2.29; 1.28-4.11), and strictures (4.62; 1.03-20.8) remained significant. The five year risk of colorectal cancer with a macroscopically normal colon was no different from that of matched general population controls.

1.2 Predicting outcome in severe ulcerative colitis

Historically, 15% of patients with ulcerative colitis will develop a severe attack of colitis requiring intensive in-patient medical therapy. In those who have developed severe disease, consistent data over the last 40 years have shown that 30-40% of patients will fail to respond to high dose intravenous corticosteroids and in-patient supportive management and require colectomy (Table 1). The mortality associated with such attacks has fallen from levels of 31-61% to the present level of 1-2% following the introduction of high dose corticosteroid therapy, better joint medical-surgical management and the acceptance of a policy of early surgery in patients not responding to medical therapy(Truelove et al. 1978). The morbidity associated with severe attacks of UC remains high and much of this is associated with delayed surgery and also prolonged exposure to high-dose corticosteroid therapy.

1.2.1 Clinical predictive factors

The current improvements in the rates of morbidity and mortality in severe UC have arisen from the development of an integrated medical-surgical care and the policy of early selection of patients requiring emergency colectomy in severe UC. These developments are catalysed by studies identifying clinical parameters and early prognostic markers associated with failure of medical therapy. Truelove and Witts initially demonstrated that the severity of presentation of UC predicted the likelihood of non-response to corticosteroid therapy. In their study involving a cohort of 210 patients with chronic UC, 10 of 31 patients (32%) with severe disease entered remission compared with 18 of 24 patients (75%) with mild disease ($p=0.075$).

Table 1: Rate of failed medical therapy in acute severe ulcerative colitis

Author	Year	Rate of medical failure (%)
Lennard Jones et al.	**1975**	**25**
Jarnerot et al.	**1985**	**40**
Chakaravarty et al.	**1993**	**28**
Travis et al.	**1996**	**29**
Lindgren et al.	**1998**	**34**
Carbonnel et al.	**2000**	**48**
Ho et al.	**2004**	**40**

Further clinical studies have therefore attempted to improve the ability to predict failure of medical therapy within the patients wuth acute severe UC. In a large cohort from St. Mark's Hospital, London; Lennard-Jones et al. examined 56 clinical features in 181 acute colitis admissions over a 5-year period, and showed that the variables most predictive of surgery were a combination of fever (>38°C) and a stool frequency of > 8/24 hours. X-ray features of colonic dilatation and mucosal islands were also associated with more severe disease(Lennard-Jones et al. 1975). The importance of serum albumin as a prognostic factor was demonstrated in a parallel publication, where the serum proteins were evaluated by the same investigators in 36 patients presenting with 39 episodes of acute severe UC(Buckell and Lennard-Jones 1979). Hypoalbuminaemia (<20g/L) and total protein concentrations <60g/L were each associated with an operative rate of >60% ($p<0.05$). A subsequent study reported a significant correlation between serum albumin levels and colonoscopic activity of UC ($r=50.48$; $p<0.05$)(Buckell et al. 1979). Chakravarty et al. reported a single centre experience involving 89 patients with acute UC treated over a 6-year period (Chakravarty 1993). In this study, the need for surgery was significantly associated with high stool frequency ($p=0.0001$) and low albumin levels (mean 30.0 vs. 38.1 g/L; $p=0.0001$).

1.2.2 Endoscopic and radiological predictors of outcome

So far, the studies described have only shown the discriminant values of simple clinical features and laboratory parameters in severe UC. There remains a question of whether radiological or endoscopic tests can be beneficial or additive to the above. It is currently accepted that the most useful radiological finding is the presence of colonic dilatation

(defined by the presence of an inflamed colon with the transverse colon or caecum dilated to 5.5 or 9 cm respectively). This finding reflects the loss of tensile strength of the colon as a result of very advanced tissue damage from inflammation leading dilatation and an increased likelihood of perforation. In some centres, colonic dilatation may itself be an indication for surgery. In addition, the development of colonic dilatation in severe UC may be heralded by abnormal small bowel distension. The presence of small bowel distension (more than 2-3 loops) has been shown to be increased in non-responders compared with responders to medical therapy in severe UC (73% vs. 43%, $p<0.05$)(Chew, Nolan, and Jewell 1991). In a further study involving 69 consecutive patients with severe UC, the presence of small bowel distension was shown to be associated with future development of colonic dilation(Caprilli et al. 1987). The presence of mucosal islands may indicate impending dilatation and disintegration, and are associated with a poor outcome(Travis et al. 1996).

In a cohort consisting of 85 patients with severe UC, Carbonnel and colleagues studied the value of combined colonoscopic findings with clinical and laboratory data(Carbonnel et al. 1996). The presence of severe colitis at endoscopy, the presence of Truelove and Witts' criteria and duration of relapse of greater than 6 weeks, were demonstrated to be significant predictors of colectomy in this study. The presence of two of these criteria would lead to 75–86% risk of colectomy within 30 days of presentation. The importance of deep ulcerations as determined endoscopically was highlighted in this study. Forty-three patients (94%) demonstrating this endoscopic feature, required emergency colectomy, with 5 (11%) patients demonstrating features of colonic dilatation.

Although the use of air, barium and air/barium enemas have been shown to be useful in detecting deep ulceration and to correlate well with histological assessment (Almer et al. 1995; Buckell et al. 1979), none of the above imaging modalities has gained widespread acceptance.

Table 2: The identification of clinical, radiological, endoscopic and biological parameters of poor outcome in severe ulcerative colitis

Author (year)	**Prognostic factors**
Truelove (1955)	Truelove and Witts' criteria
Lennard-Jones (1975)	Fever >38°C + >8 stools/day at 24h Colonic dilatation and hypoalbuminaemia
Buckell (1979)	Hypoalbuminaenia
Buckell (1980)	Colonic dilatation on AXR
Chew (1991)	Small bowel distension, pan-colitis on AXR
Almer (1985)	Ulcers on air enema, fever >380C, low albumin
Carbonnel (2000)	Severe endoscopic colitis, relapse>6 weeks, Truelove and Witts' criteria
Chakravarty (1993)	Stool frequency, hypoalbuminaemia
Travis (1996)	Stool frequency >8, or 3-8 stools and CRP $\geq$ 25 mg/l at day 3
Lindgren (1998)	Fever for >24h, day 3 stools >4 and CRP $\geq$ 25 mg/L
Rowe (2000)	High percentage of band neutrophils on admission

1.2.3 Statistical modelling in predicting outcome

Although the identification of clinical and endoscopic/radiological features of poor outcome have been forthcoming, the translational challenge has lied in the assimilation of these features into a clinically applicable model which allows for the ***early*** identification of patients who are at high risk of failure to respond to medical therapy. This has important implications in counselling and the reduction of morbidity/mortality which are associated with delayed surgery and prolonged exposure to high-dose corticosteroid therapy. The current British Society of Gastroenterology guidelines recommend the objective evaluation of all patients with acute severe UC at day 3 of intravenous corticosteroid therapy, to determine which patients will benefit early surgery(Carter, Lobo, and Travis 2004).

Two models have been described in the literature to aid such clinical-decision making, e.g. to select patients for early colectomy – the Travis criteria and the Lindgren model(Travis et al. 1996; Lindgren et al. 1998). In a prospective study involving 49 patients with severe UC, Travis and colleagues proposed that 85% of patients with a stool frequency of >8/day or 3-8/day with C-reactive protein of 45 mg/dl after 3 days of intravenous corticosteroid therapy will fail to respond and require colectomy(Travis et al. 1996). During the first five days of medical therapy, stool frequency and C reactive protein (CRP) distinguished between outcomes of surgery and early response ($p < 0.00625$). Lindgren and colleagues developed a regressional formulae to predict the likelihood of medical failure - *number of bowel movements* $+0.14\times$ *CRP (mg/l)*>8.0, as

the optimum theoretical cut-off level to predict failure of corticosteroid therapy(Lindgren et al. 1998). Patients with a score of >8, has a 75% chance of requiring colectomy.

The clinical focus of the prediction of patients requiring early surgery using the above two models has changed with the expansion of effective 2nd line medical therapy, pertinently, ciclosporin and infliximab(Lichtiger et al. 1994; Jarnerot et al. 2005b). It is now accepted that a proportion of patients will respond very favourably to intravenous corticosteroids – and in the group of patients who do not, the dilemma arises in which patients to treat with second-line medical therapy or early surgery. The existing models by Travis and Lindgren do not critically assist decision-making in the selection of these patients. For example, the Lindgren model, together with a further activity index (Seo-index) has been applied in a Swedish-Danish study in assessing the efficacy of infliximab in acute UC. The Seo-index is calculated at using the following formula: 60 x blood in faeces + 13 x bowel movements/day + 0.5 x ESR - 0.4 x Hb (g/l) - 1.5 x albumin (g/l) + 200. Constants used are for blood in faeces: 0 = none, 1 = present and for bowel movements 0 - 3 = 0, 4 = 1, 5-7 = 2, $\geq$ 8 = 3. A value < 150 corresponds to remission or mild UC, 150-220 moderately severe UC and > 220 severe UC. Only the Seo-index appeared to be beneficial in identifying a subset of patient who may benefit from infliximab at a later stage (days 5-7). In patients with low Seo-index, no patients treated with infliximab compared with 62.5 % in the placebo group required a colectomy (p=0.009). In this study, the Lindgren model (calculated at day 3) was not useful and again highlighted the difficulties in the selection of patients who can benefit from early 2nd line medical therapy.

1.3 Pathogenesis of inflammatory bowel disease

Both human and animal studies suggest that the intestinal inflammation in ulcerative colitis are likely to be a consequence of environmental factors triggering a breakdown in the regulatory constraints on mucosal immune responses to enteric bacteria in genetically susceptible individuals(Fiocchi 1998; Podolsky 2002). The relatively weaker concordance in monozygotic twin pairs with ulcerative colitis (6–14%) compared with Crohn's disease (44–50%) suggest that environmental factors are more important than genetic factors in the pathogenesis of ulcerative colitis (Orholm et al. 2000; Tysk et al. 1988b). Cigarette smoking and appendectomy are the two best-characterized environmental factors. The protective effect of smoking (and the detrimental effect on Crohn's disease) is the most consistent environmental factor within IBD. In a study by Lindberg et al., the relative risks of developing ulcerative colitis in heavy ex-smokers, all ex-smokers, non-smokers, and smokers were 4.4, 2.5, 1.0, and 0.6, respectively(Lindberg et al. 1988). In the case for appendectomy, thirteen published studies summarised by a recent meta-analysis have consistently demonstrated a protective effect of appendectomy against the development of UC(Koutroubakis and Vlachonikolis 2000).

1.3.1 Evidence of genetic susceptibility in disease pathogenesis

There are considerable epidemiological data, which implicate genetic susceptibility in the pathogenesis of Crohn's disease and ulcerative colitis. Most notably these include the familial prevalence of inflammatory bowel disease, concordance rates in twin pairs, and ethnic differences in disease susceptibility. In fact, the studies of twin pairs have provided the strongest impetus towards further investigation of genetic susceptibility in inflammatory bowel disease. Three studies have been carried out in Europe, including Tysk's important review of the Swedish Twin Registry in 1988(Tysk et al. 1988a; Thompson et al. 1996; Orholm et al. 2000). The data from these studies, in Sweden, Denmark and the United Kingdom combine to provide powerful evidence for the role of both genetic and environmental factors in disease susceptibility. The concordance rates for Crohn's disease in monozygotic and dizygotic twin pairs from these studies are estimated as, respectively 37% and 7%; in ulcerative colitis, the equivalent results are 10% and 3%.

From these data, the coefficient of heritability (an estimate of the proportion of susceptibility attributable to inherited factors alone) may be calculated. The derived coefficient of heritability in Crohn's disease is similar to the value calculated from twin studies carried out in insulin dependent diabetes and multiple sclerosis, and in fact is stronger that many other common complex diseases. The genetic contribution is clearly less strong in ulcerative colitis, but the concordance between monozygotic and dizygotic prevalence rates again argues for a genetic and perhaps a stronger relative environmental contribution to disease susceptibility in ulcerative colitis.

A large number of studies have now been reported, investigating the familial aggregation of inflammatory bowel disease. Overall, it is apparent that between 6 and 32% of patients with inflammatory bowel disease have an affected first or second-degree relative. The prevalence of a positive family history is highest in certain groups – risk factors for familial inflammatory bowel disease include Jewish ancestry, and early onset disease (Orholm et al. 1991; Roth et al. 1989; Halfvarson et al. 2003; Monsen et al. 1991; Probert et al. 1993). These observations in themselves are important, suggesting that these subgroups of inflammatory bowel disease may have a particularly strong genetic contribution. The exact size of the relative risk to family members of patients with Crohn's disease and ulcerative colitis varies from one study to another. This may reflect the study design, or effects of ethnic and phenotypic variability on genetic contribution. However, consistent themes are present. The relative most commonly affected is the sibling of the patient with inflammatory bowel disease. Parents, offspring and second-degree relatives appear to have a lower risk. The data, which have further excited geneticists, have been the relative risk to siblings (lambda S) in Crohn's disease and ulcerative colitis. This ratio has been estimated as between 13 and 36 in Crohn's disease, and between 7 and 17 in ulcerative colitis. Equivalent figures in type I diabetes, schizophrenia and cystic fibrosis are 15, 8.6, and 400 respectively.

The development of a linkage map of the human genome with informative microsatellite markers has enabled hypothesis-free scanning of the human genome for loci associated with susceptibility to simple monogenic and polygenic diseases. In the recent years, significant progress has been achieved using molecular data from genome-wide linkage studies of multiply affected IBD families. Genome wide scanning has identified susceptibility loci for Crohn's disease on chromosomes 1 (Cho et al. 1998b), 5 (IBD5)(Rioux et al. 2000a; Rioux et al. 2001b), 6 (IBD3; HLA)(Hampe et al. 1999a; Rioux et al. 2000b), 12 (IBD2)(Satsangi et al. 1996a), 14 (IBD4) (Ma et al. 1999b; Duerr et al. 2000b), 16 (IBD1)(Hugot et al. 1996a; Satsangi et al. 1996c) and 19 (IBD6) (Rioux et al. 2000c). The most consistently replicated Crohn's disease susceptibility locus is located on chromosome 16 (IBD1) and the susceptibility gene has been identified as the CARD15 (NOD2) gene (Hugot et al. 2001b; Ogura et al. 2001a). These studies have been remarkably successful in identifying a number of susceptibility loci, with convincing replication shown for at least 7 loci (IBD1-7) (Table 3). Until 2006, the success of identifying genes that confer susceptibility to IBD has been based on the platform of the data generated from these genome-wide linkage studies, which is discussed in the following chapter. The publication of genome wide association studies (GWAS) which utilised up to 500 000 SNPs per individual represents a further shift in the progress of IBD genetics.

Table 3: Major susceptibility loci identified by genome screening

IBD locus designation	Chromosomal location	Study	Diagnoses
IBD1	16q12	(Hugot et al. 1996b)	CD
IBD2	12q13	(Satsangi et al. 1996b)	UC
IBD3	6p13	(Hampe et al. 1999b)	CD, UC
IBD4	14q11	(Ma et al. 1999a), (Duerr et al. 2000a)	CD
IBD5	5q31-33	(Rioux et al. 2001a)	CD
IBD6	19p13	(Rioux et al. 2000d)	CD, UC
Other loci	1p36	(Cho et al. 1998a)	CD, UC
Other loci	7q22	(Satsangi et al. 1996b)	CD, UC
Other loci	3p	(Satsangi et al. 1996b)	CD, UC

1.3.2 Genetics of Crohn's disease: from linkage to gene discovery

NOD2/CARD15 gene

Following the success of genome-wide scan studies in identifying chromosomal locus of disease susceptibility, significant progress has been made particularly in Crohn's disease. In 2001, 3 independent groups reported the identification of the first Crohn's disease (CD) susceptibility gene, NOD2 (subsequently renamed *CARD15* by the International Nomenclature Committee), on chromosome 16q12 (the IBD1 locus)(Ogura et al. 2001b; Hugot et al. 2001a; Hampe et al. 2001). This breakthrough firmly established a role for genetics in determining susceptibility to CD and has provided proof of principle that model-free linkage analyses may be used successfully to identify disease susceptibility gene loci.

Hugot and colleagues employing a classical positional cloning strategy refined the IBD1 susceptibility locus by genotyping 26 microsatellite markers spaced at an average distance of 1 centimorgan in the pericentromeric region of chromosome 16, in 77 multiply affected families. Further genotyping and sequence analysis led to the identification of a single gene, the now known NOD2/CARD15 gene. Within this gene, 3 SNPs showed significant evidence for linkage disequilibrium, and association with Crohn's disease. These findings were also shown in a parallel publication by Ogura and colleagues; these investigators utilized a candidate gene approach based on prior knowledge of the chromosomal location of NOD2/CARD15, and encouraged by functional and expression data implicating NOD2/CARD15 as a possible candidate in disease pathophysiology. In addition, Hampe and colleagues reported the association of

the 3020insC frameshift mutation with Crohn's disease in German and British populations using parallel strategies of a family based association study, and case control study. The authors demonstrated genotypes specific disease risks of 2.6 (heterozygous genotype) and 42.1 (homozygous for the mutant 3020insC frameshift NOD2/CARD15 mutant allele).

Recent studies have further shown a number of associations between genotype and phenotype(Ahmad et al. 2002; Cuthbert et al. 2002), thus adding to the complexities of IBD and reinforcing the point that the term, IBD may in fact represent several forms of diseases. Cuthbert and colleagues demonstrated the association between NOD2/CARD15 mutations and IBD are primarily based on the phenotype of ileal Crohn's disease. This association was also shown in the Oxford dataset by Ahmad and colleagues who reported detail genotype-phenotype analysis of 244 patients with Crohn's disease. The phenotype of fibro-stenosing Crohn's disease was also associated with NOD2/CARD15 mutations, but this association was not shown to be independent of the association with ileal involvement. The NOD2/CARD15 gene does not appear to be associated with colonic involvement, and may indeed be protective against colonic involvement in Crohn's disease and ulcerative colitis.

The association of NOD2/CARD15 gene with CD also highlighted yet a further important point – the importance of genetic heterogeneity. Reported NOD2/CARD15 carriage rates in Crohn's disease vary between 0 and 50.0%, with highest rates seen in central European populations (Hugot et al. 2001c; Lesage et al. 2002) while mutations are

absent in Japanese and Chinese series (Inoue et al. 2002; Leong et al. 2003). Lower frequencies have been reported from Finland, Ireland and Scotland suggesting further heterogeneity even within Europe (Arnott et al. 2004b; Helio et al. 2003). This would suggest that the genetic contribution of NOD2/CARD15 gene is less significant for some populations, and that other genes maybe pertient in disease aetiopathogenesis.

The NOD2/CARD15 undoubtedly is the most successful example of progress from linkage (IBD1 linkage region in chromosome 16) to gene discovery. In CD, other progress has been forthcoming with postulated implicated genes of *organic cation transporter*, OCTN1/2 (Peltekova et al. 2004) and *drosophila large disc*, DLG5 (Stoll et al. 2004) genes in the IBD5 (chromosome 5q22) and IBD4 (chromosome 14q) regions respectively. The roles of these genes however, have not been fully defined and the associations have been inconsistent. The effects of linkage, genetic and phenotypic heterogeneity are likely to explain some of these inconsistencies. Some authorities have suggested that the size effect of NOD2/CARD15 gene is likely to be the ceiling of the genetic contribution in IBD e.g. the genetic contributions of other genes will be significantly less.

1.3.3 Genomewide association studies

With the availability and the increasing economy in high-throughput genotyping, coupled with the knowledge of linkage disequilibrium and complete genomic variations; the ability to catalogue the majority of the polymorphisms (300 000-500 000 SNPs using array-based technology) in large case-control association study settings is now feasible.

In 2007, genomewide association studies have been remarkably successful in Crohn's disease, with the identification at the time of writing of 30 confirmed susceptibility genes or loci yielding major pathogenic insights. These studies have most importantly implicated hithertho unknown novel mechanisms involving the Th17 (in interleukin receptor 23 *IL23R* gene) and autophagy pathways (the process of self cellular digestion). Duerr et al. observed a highly significant association between Crohn's disease and the interleukin receptor 23, *IL23R* gene on chromosome 1p31, which encodes a subunit of the receptor for the proinflammatory cytokine interleukin-23 in an enriched cohort of ileal CD (Duerr RH et al. 2006). This approach was used in order to address phenotypic heterogeneity. In this study, three markers achieved genome-wide significance for which 2 of these were within the NOD2/CARD15 gene. The third marker, rs11209026 (P $=5.05\text{x}10^{-9}$, corrected P $=1.56$ x 10^{-3}), is a nonsynonymous SNP (c.1142G>A, p.Arg381Gln) in the *IL23R* gene (GenBank accession: NM_144701 [GenBank] , GeneID: 149233) on chromosome 1p31. This gene encodes a subunit of the receptor for the proinflammatory cytokine, interleukin-23 (IL-23), and is therefore an intriguing functional candidate. In addition to Arg381Gln, nine other markers in *IL23R* and in the intergenic region between *IL23R* and the adjacent IL-12 receptor, beta-2 gene (*IL12RB2*), had association P-values < 0.0001 in the non-Jewish, ileal CD case-control cohort. It is of interest to note that a further uncommon coding variant (rs11209026, c.1142G>A, p.Arg381Gln) confers strong protection against Crohn's disease. Replication studies confirmed *IL23R* associations in independent cohorts of patients with Crohn's disease or ulcerative colitis. These results and previous studies on the proinflammatory role of IL-23 prioritize this signaling pathway as a therapeutic target in inflammatory bowel disease.

The presence of a protective variant also imply that the contribution of the *IL23R* pathway to IBD will likely involve more than simple gain- or loss-of-function *IL23R* variants, and therapeutic interventions will be improved by a better understanding of the context and tissue-specific events associated with functional *IL23R* polymorphisms.

Hampe and colleagues analysed approximately 20,000 coding SNPs (SNPlex Genotyping system) in a North German cohort of 735 CD patients and 368 healthy controls (Hampe, 2007). 7,159 out of the 16,360 successful assays had a minor allelic frequency of >1%. On allele-based testing for association 72 of these 7,159 were significant at $p<0.01$. These SNPs were then tested in a second panel consisting 380 CD trios, 498 CD singletons and 1,032 healthy controls. 3/72 SNPs satisfied a pre-defined statistical cut-off of $p<0.05$: rs2066845 in *CARD15* (rs206684 failed genotyping; rs2066847 was not feasible on SNPlex due to the necessary insertion design), rs1050152 (L503F) in *SLC22A4*, and rs2241880 (T300A) in *ATG16L1* (autophagy-related 16-like 1 gene) on chromosome 2q37 near a locus of susceptibility (case-control $p=1.6 \times 10^{-5}$; TDT $p=2.7 \times 10^{-5}$). After re-sequencing of *ATG16L1* in 47 CD samples failed to detect any further coding or splice site variants, 28 tagging SNPs ($r^2 > 0.8$; MAF > 1%) were selected from the CEU HapMap data. The results of logistic regression and haplotype analysis suggested that the CD risk from *ATG16L1* was confined to the G allele of rs241880. Analysis of the combined cohorts gave an odds ratio of 1.45 (95% C.I. 1.21-1.74) and population attributable risk (PAR) of 0.26 for carriage of this variant, with some evidence for a gene dosage effect (homozygote OR 1.77, C.I. 1.43-2.18; PAR 0.17). There was no specific sub-phenotype in CD associated with this variant, and no association with UC.

There was, however, a statistical interaction between rs2241880 and *CARD15* low-risk haplotypes (p=0.039).

The Wellcome Trust Case Control Consortium (WTCCC) undertaken as a major collaborative effort involving 14 000 individuals in 7 major common complex diseases with 3 000 shared control in United Kingdom, utilizing genomewide association study has now provided remarkable insights into the genetic architecture and new mechanisms in disease pathogenesis (Crohn's disease, bipolar disorder, coronary artery disease, hypertension, rheumatoid arthritis, Type 1 and 2 diabetes)(WTCC Nature). The Crohn's dataset which represents the largest study group to date, was based on cohorts from Edinburgh, Oxford, Cambridge, London and Newcastle (1748 CD). This study confirmed previous associations with *CARD15* (rs17221417 $p=9.4x10^{-12}$), *IL23R* (rs11805303 $p=6.5x10^{-13}$) and *ATGL16* (rs10210302; $p=5.7 \times 10^{-14}$) genes in addition to several new loci with genome-wide significance such as immunity related guanosine triphosphatase, *IRGM* ($p=5.1x10^{-8}$), bassoon, *BSN* ($p=1.4x10^{-8}$), NK2 transcription factor related, locus 3, *NKX2-3* ($p=7.7x10^{-7}$) and protein tyrosine phosphatase, nonreceptor type 2, *PTPN2* genes ($p=4.6x10^{-8}$). Parkes and colleagues have subsequently genotyped 37 SNPs from 31 distinct loci, associated at significance level of $p<10^{-5}$ on initial analysis of the WTCCC dataset. Among new loci with genome-wide significance in the WTCCC scan, the strongest replication adjacent to a known gene was for SNPs rs13361189 and rs4958847 ($P_{rep}=6.6x10^{-4}$ and $3.1x10^{-4}$, respectively) immediately flanking *IRGM* on chromosome 5q33.1. Association in the combined panels of 2,930 cases and 4,962 controls was highly significant ($P_{comb} =2.1x10^{-10}$ and 3.8×10^{-9}) together with replication for nine other loci,

including NKX2-3, PTPN2 and gene deserts on chromosomes 1q and 5p13(Parkes et al. 2007).

Two further studies which were published prior to the WTCCC dataset (North American and European datasets) also demonstrated highly consistent findings. Rioux et al. utilised a cohort comprising of ileal Crohn disease with a further two independent replication cohort also confirmed established *CARD15, ATGL16* and *IL23R* genetic associations, and novel loci which with significantly replicated associations (combined $P < 10^{-10}$); in the intergenic region on 10q21.1 and genomic regions encoding *PHOX2B*, *NCF4* and a predicted gene on 16q24.1 (*FAM92B*)(Rioux et al. 2007). In a Belgian CD scan, Libioulle et al., also confirmed associations with *CARD15* and IL23R, in addition to a 1.2-Mb region of chromosome 5p13.1 that contains no known genes(Libioulle et al. 2007).

These highly significant genetic evidence regarding *IRGM, ATG16L1* (converging on autophagy pathways), *CARD15* and *IL23R* collectively implicates defects in the early immune response, particularly innate immune pathways and the handling of intracellular bacteria, in the pathogenesis of Crohn's disease. Whether the latter is restricted to a single pathogen (or, more likely, to a class of subpathogenic bacteria relying on defective host innate immunity for survival) awaits further investigation. A recent meta-analysis of the published GWAS studies has now shown more than 30 novel loci will provide further tantalising clues to the pathogenesis of CD (Barrett et al. submitted to Nature 2008).

1.3.4 Genetics of ulcerative colitis

The current evidence for the genetic contribution to the disease susceptibility is weaker in UC compared with CD. It is conceivable that the genetic contribution in UC may be more of a modifying effect to environmental stimuli rather than directly causally linked (as in CD). Although most current linkage studies contained relatively more CD-CD affected relative pairs (hence linkage regions have shown relatively greater evidence for CD compared with UC), several loci demonstrate a stronger evidence of linkage with UC compared with CD. These loci are 12q13 (IBD2), 6p13 (IBD3, HLA region) and 7q22 (Satsangi et al. 1996b; Duerr et al. 1998).

Many of the early studies focused on the highly polymorphic region, the HLA locus on chromosome 6p13, provided conflicting results, possibly due to a lack of statistical power and because typing was done serologically rather than by direct genotyping. Only one consistently replicated finding has been associated with UC. The HLA DRB1*0103 allele situated at the HLA Class II region has been shown to be associated with extensive disease, the need for surgery and extraintestinal manifestation in the Caucasian population. Although a rare allele in the general population, this association has been consistently replicated in at least three independent studies and a meta analysis(Bouma et al. 1999; Roussomoustakaki et al. 1997; Duerr and Chesny IJ 1997). While the allele is only carried by 1-3% of the general population, carriage is 7.4-11% in UC patients with extensive or pan-colitis. Furthermore, in a study of patients who have undergone surgery and ileo-anal anastamosis for severe UC, HLA DRB1*0103 allele was present in 15.8% of patients with extensive colitis at time of surgery, and in 22.8% of a subset of patients

who also had extraintestinal manifestations (Roussomoustakaki et al. 1997). An association between the HLA DRB1*1502 (DR2) with UC has also been observed in several studies, notably in USA and Japan(Toyoda et al. 1993; Futami et al. 1995; Sugimura et al. 1993). Moreover, in the Japanese population, HLA DRB1*1502 (a sub-group of DR1*15) is common and has been associated with intractable disease. HLA-DR4 allele has been shown in several studies to be protective against UC, and this was confirmed in a meta-analysis by Stokkers et al.

The contribution of the HLA Class I and III regions have received less attention than the Class II determinants. The tumour necrosis factor-α (TNF- α) genes are located within the major histocompatibility region and IBD3 linkage interval on chromosome 6p. Koss and colleagues have suggested that TNF-2 haplotype maybe predictive of disease extent (Koss et al. 2000). However, this observation may simply reflect linkage disequilibrium within the HLA region. De La Concha et al. have recently demonstrated that susceptibility to severe UC may be associated with polymorphism of the IKB-like (IKBL) gene(de la Concha et al. 2000). The IKBL +738C allele lies in tight linkage disequilibrium with HLA DRB1*0103-these two allelic variants were significantly increased in individuals with severe UC. Nevertheless, a significant association was also observed in HLA DRB1*0103 negative patients. These findings have not been replicated yet to date.

Mansfield et al. found that the carriage of the less common allele 2 of the interleukin-1 receptor antagonist (IL-1RA) was increased in patients with extensive UC (35% vs. 24%

in controls) (Mansfield et al. 1994). However, this association has not been uniformly replicated (Bouma et al. 1996b; Tountas et al. 1999). A larger study from the same center by Carter and colleagues confirmed a minor association conferring a small risk, especially in patients with extensive disease (Carter et al. 2001a). A subsequent study by the same author reported on IL-1RA allele 2 carriage in 109 patients with UC treated with colectomy. The investigators suggested that the rate of allele 2 carriage might be predictive of the development of inflammation within the ileo-anal pouch (pouchitis) (Carter et al. 2001a). However, a larger study has again questioned any association of IL-1RA with IBD (Aithal et al. 2001).

Cytokines play a crucial role in the initiation and regulation of the immune response(Louis et al. 1996). The genes that encode these proteins by virtue of their function make these attractive candidate genes for susceptibility, and perhaps, severity, to UC. Nevertheless, studies to date have not yet provided a consistent evidence of a strong effect on disease susceptibility or severity(Mansfield et al. 1994; Carter et al. 2001a; Carter et al. 2001b; Olavesen et al. 2000; Parkes, Satsangi, and Jewell 1998b; Aithal et al. 2001; Klein et al. 2001). Others studies looking at the effect of cytokine polymorphisms, such as IL-10, IL-6, IL-4, and IL-2,(Olavesen et al. 2000; Parkes, Satsangi, and Jewell 1998a) have not yet provided consistent evidence of a strong effect on disease susceptibility or severity (Aithal et al. 2001).

There have been suggestions that genetic markers may be useful in predicting the course of disease in UC. Recent data presented in abstract form by McGovern et al. suggest that

a panel of genetic markers may be useful in predicting colectomy in UC. The authors utilized a combination of markers involving 3 genes, MEKK1 (MAP3K), MDR1 and the HLA DRB1*0103 allele – and showed using a Bayesian approach the odds of prediction can be significantly improved. Nevertheless, no further insights have been shed on the contribution of these genes to pathophysiology of disease and given the clear evidence of both phenotypic and genetic heterogeneity; these data has yet to be shown to be of value in clinical practice. Achkar et al. in an IBD linkage study involving 904 affected relative pairs also provided strong linkage evidence for the subset of extensive ulcerative colitis in the region of IBD2 (lod 3.27; $p < 0.001$)(Achkar et al. 2006).

Recently, inherited variations of myosin IX (MYOB9B) gene have also been shown to be associated with UC in three independent populations(van Bodegraven et al. 2006). It is of interest to note that the initial association was primarily observed with coeliac disease in the Dutch population (Monsuur et al. 2005). The mechanism underlying this is unclear. Overexpression of rat myosin IXB leads to actin filament related morphological changes in epithelial cells, and human myosin IXB is expressed in intestinal epithelial cells. It is postulated that variations of the MYOB9 gene may influence intestinal permeability leading to inflammatory intestinal disease susceptibility. As with the recent progress with CD, the results of genome-wide association studies in UC are anticipated to reveal significant new insights into the disease pathogenesis of UC.

1.3.5 Association studies and linkage disequilibrium

An influential paper by Risch and Merikangas suggested that in complex diseases where the genetic contribution is only modest (low penetrance), direct examination of the risk variant itself using for example, a case control study can be more powerful than the examination of the indirect effect of a risk factor has on allele sharing among siblings, in the case of linkage studies(Risch and Merikangas 1996). Two experiences have led to a broader acceptance of the strategy proposed therein. Firstly, highly significant linkage results are very uncommon in common diseases (despite numerous genome scans in many diseases and some considerably large meta-analysis in a number of diseases such as Type 2 diabetes and multiple sclerosis) suggesting that a major high-penetrance risk factor is not typical in most common diseases. Secondly, recent positive results in a number of diseases suggest that common genetic variations with a modest associated disease risk does have a significant role in the contribution to disease susceptibility (Stefansson et al. 2002; Strittmatter et al. 1994; Ueda et al. 2003; Altshuler et al. 2000). The latter point lend further support to the now widely-described, 'Frequent disease-frequent variant hypothesis', which suggest that the genetic factors predisposing to, or associated with a disease of high prevalence should also be frequent in the same population.

Studies of common diseases have fallen into two broad categories: family-based linkage studies across the entire genome, and population-based association studies of individual candidate genes. Although there have been notable successes, progress has been slow due

to the inherent limitations of the methods; linkage analysis has low power except when a single locus explains a substantial fraction of disease. The earlier association studies relies on the selection of candidate SNPs based on location and on the basis of their respective effects in coding changes within exonic or promoter sequences that may have biological or functional consequence. This approach examines only a small fraction of the 'universe' of sequence variation in each patient.

The arrival of denser SNP maps and the completion of the International HapMap project, it is now possible to determine the patterns of *linkage disequilibrium* of genomic sequences(A haplotype map of the human genome 2005). Linkage disequilibrium is the non-random association between alleles at different loci with a particular combination of alleles along a chromosome is termed a *haplotype*. In some cases, an allele at one locus predicts which allele which would be found in another locus, making one of the loci redundant in mapping purposes. Thus in principle, with the knowledge of LD of a particular genomic region or gene, it is now possible to identify and genotype only a small number of SNPs which would represent the variations from that region or gene, thus allowing the possibility of examining a very larger number of genes with high throughput genotyping with very good economy of effort. Nevertheless, this knowledge has introduced a further difficulty in the interpretation of earlier association studies which were implicitly candidate polymorphism rather than candidate gene studies. Failure of a candidate-polymorphism study does not rule out involvement of the gene or genes. The selection of highly informative SNPs (tagging SNPs) is critical in the validity of an association study, and is influenced by many factors as discussed in the following sub-

chapter. A negative candidate-gene study can provide a statistical limit to the importance of any common variant in the gene. However, there remain practical difficulties in demonstrating causality of SNPs once association is achieved(Trinh and Rioux 2005).

1.3.6 Haplotype-tagging approach

The concept of haplotype-tagging is based on the knowledge that the human genome is composed of long tracts of limited diversity (haplotype 'blocks') interspersed by areas of recombination ('hotspots')(Gabriel et al. 2002). In these haplotype blocks, it is considered that redundant information exist even amongst densely spaced SNP markers, and usually a subset of markers can retain all or most of the information in the region studied. The recently completed International HapMap Project has now confirmed the generality of hotspots of recombination, with long segments of strong LD which displayed limited haplotype diversity(A haplotype map of the human genome 2005). The confirmation of the extensive redundancy of SNPs has lead to the potential to extract extensive information about genomic variation without complete resequencing, the development of selection methods of tag SNPs and the optimization of association analyses. Current thinking about the design of association studies has been greatly influenced by the above data, where the common haplotypes can be represented by knowledge of only a few genotypes in the block, with stretches of more rapid breakdown of linkage disequilibrium, where a few sentinel SNPs cannot reliably represent haplotype diversity; in many cases, the latter regions correspond to hot spots of meiotic recombination(Daly et al. 2001). This structure of linkage disequilibrium inspired the idea

of haplotype tagging, in which a set of SNPs is identified that tags each of the common haplotypes within a block of linkage disequilibrium.

Since the seminal paper by Johnson et al. describing the haplotype-tagging approach in 2001(Johnson et al. 2001), many different statistical methods and algorithms have been developed(Zeggini et al. 2005). These methods usually differ on one of the following aspects:

- Source of genetic information
    - Haplotype
    - Genotype
- Measure and criteria of evaluation in tSNP evaluation
    - Haplotype diversity
    - Haplotype r^2
    - Pairwise r^2
    - Entropy
    - Combination approach
- Computing algorithms
    - Exhaustive search
    - Analytical and heuristic operations
    - Hierachical clustering and graph methods
    - Greedy algorithms and dynamic programming

Despite these differences, the basic template of approach is the same. Firstly, a representative sample of a population is extensively sequenced to provide the reference data; and the extent of linkage disequilibrium is assessed. Secondly, a set of SNPs is selected to represent the variations of the gene/region studied. These SNPs are then genotyped in a case-control or a family based study cohort. Such association raises 2 fundamental questions: a) how well the selected SNPs represent the undetected variations in the original sample and b) how well these SNPs will represent variation (both detected and undetected in a new sample from the same population?

Several studies have now examined this issue and demonstrated no obvious discrepancies in terms of the efficiency and effectiveness in these methods(Ke et al. 2005; de Bakker et al. 2005; Burkett et al. 2005). Given practical limitations on genotyping, investigators are forced to make several practical decisions: (i) selecting and prioritizing tag SNPs (ii) deciding which tests of association to use and (iii) evaluating statistical significance of putative findings. Genotyping a higher density of tag SNPs increases the fraction of sites captured through LD, but the quantitative relationship between additional genotyping and increased power in association studies is not well described. The use of multimarker haplotypes shifts this relationship toward greater efficiency(Goldstein et al. 2003) has certain drawbacks: if haplotype testing increases the degrees of freedom or number of tests in statistical analysis, it may decrease, rather than increase, overall power(Chapman et al. 2003). Many studies will rely on data from the International HapMap Project, which is an extensive but incomplete inventory of common genetic variation(A haplotype map of the human genome 2005). Recently De Bakker et al. demonstrated that specified

multimarker tests substantially increase tagging efficiency relative to single-marker approaches, without loss of power(de Bakker et al. 2005). Second, when selecting SNPs from very dense reference panels, a method such as the best *N* strategy, which ranks SNPs according to the number of proxies they have, allows marked reductions in genotyping with limited loss of power, substantially outperforming a method based on relaxing r^2 thresholds. Third, sparser sets of tags selected from a pseudo phase I HapMap are almost as powerful as equally sized sets chosen from complete reference panels. Fourth, exhaustive multimarker tests improve power for less common causal alleles but are neutral or reduce power when the causal SNP is common. These relationships hold for each of the different population samples studied by HapMap, although the number and performance of tags varies, as expected, according to the general extent of LD in each sample.

Haplotype tagging: haplotype r^2 method

At its essential core, the most direct approach to identify a set of tSNPs that maintains a high r^2 value directly with other (tagged) SNPs (or to define a set of tSNPs that do so). r^2 values can be defined as coefficients of determination (i.e. the proportion of explained variations) obtained from fitting linear models predicting the state of tagged SNPs from the set of tSNPs. For haplotype r^2, the model is fitted to the G haplotypes defined by:

$$\mathbf{Y_i} = \mathbf{x_i b_1} + \mathbf{x i_2 b_2} + \ldots\ldots \mathbf{x_{iG} b_G}.$$

Where $\mathbf{Y_i}$ is the predicted state of the tagged SNP of interest on the $_i$th chromosome (e.g. representing observed values as 0 or 1), $\mathbf{x_i}\ldots\ldots\mathbf{x_{iG}}$ are indicator variables (0 or 1 depending on which haplotypes are present) and $\mathbf{b_1}\ldots\ldots\mathbf{b_G}$ are coefficients estimated by standard least

squares from the observed data. Pairwise r^2 values between two SNPs (or between aSNP and the presence or absence of a single haplotype) can be defined by setting G = 2. If the tagged SNP state or the haplotype state on chromosome ***i*** are not known with certainty, weighted least squares can be used, either by splitting each observed chromosome into all possible SNP and haplotype states with weights equal to their estimated probabilities, or equivalently by listing all SNP and haplotype combinations consistent with the data and using weights equal to their estimated population frequencies (e.g. from EM estimation). Software for the fitting and evaluation of r^2 values is available in the TagIT package (http://popgen.biol.ucl.ac.uk/software.html).

Pritchard and Przeworski showed, under simplifying assumptions and when G=2, that the power of a case-control study comprising ***n*** observations looking directly at a causal SNP was equivalent to the power of a case-control study comprising n/r^2 observations looking at a marker, where r^2 is the coefficient of determination between the marker and the causal SNP. We have verified this simple power relationship using simulations, and have also found that the relationship holds when G>2 (M.E.W., unpublished). To illustrate these approaches, Goldstein and colleagues identified four tSNPs for region 25b in the Gabriel et al. dataset using different criteria. The 4 tSNPs for the European sample provide a haplotype r^2 of 0.96 or above for each of the seven 'tagged' SNPs in this region. Many of the tagged SNPs in this region, however, fail to show high pairwise r^2 values with any single SNP. For example, the tSNPs are SNPs 4, 5, 8 and 11 in order through the region. For tagged SNP 1, however, the pairwise r^2 values against the tSNPs are 0.32, 0.08, 0.02, and 0.05 (Figure I). Similarly, the pairwise r^2 values between the

haplotypes defined by these tags and the tagged SNPs do not perform better; they are all below our cut-off of 0.85. These comparisons suggest that in the selection of tagging SNPs, and in the subsequent association study, it might be necessary to consider association tests that either use a regression model with haplotypes as independent predictors or employ other approaches which consider clusterings of haplotypes. After selecting a set of tSNPs, it is necessary to assess how well they represent as yet undetected SNPs. The terminology of Weale and Goldstein defined K as the total number of SNPs identified in the region of interest, H as the tSNPs identified from K, and A the set of all common SNPs in the region (http://popgen.biol.ucl.ac.uk/software). The question to answer is how well H represents the set of SNPs in A. To assess this, we have used a sub-sampling procedure in which we sequentially drop out SNPs from the set K and identify a set of tSNPs in the reduced set. The performance of the tSNPs in predicting the state of each of the dropped SNPs in turn, and so estimate the performance against the (unknown) elements of A is then subsequently checked. The haplotype r2 tagging method is used in the association studies described in this thesis.

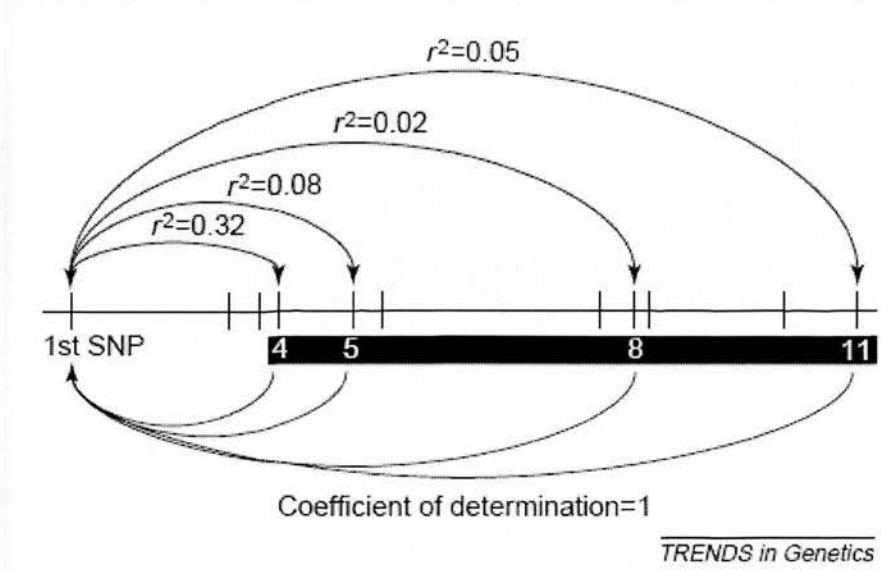


Fig. I. Illustration of relative SNP positions and pairwise r^2 values for the 11 SNPs in region 25b of the Gabriel *et al.* [13] dataset, as described in Box 1. The tSNPs for this region are SNPs 4,5, 8 and 11. As shown, none has a high pairwise r^2 against SNP 1, although SNP 1, like the other SNPs, is well predicted by the haplotype r^2 criterion. Abbreviations: SNP, single nucleotide polymorphism; tSNP, tagging single nucleotide polymorphism.

Figure 1: Illustration of relative SNP positions and r2 values (adapted from Goldstein et al. Trends in Genetics).

1.4.1 Mucosal immunology and luminal flora in ulcerative colitis

Populations of mucosal B cells and plasma cells increase in ulcerative colitis, which initially suggested that the disease was antibody mediated and complement dependent (Halstensen, Das, and Brandtzaeg 1995). However, the main abnormality driving the inflammation is now considered to be secondary to an exaggerated T-cell response causing mucosal hyper-responsiveness to commensal bacteria(Sartor 2006). The B-cell response and increased production of IgG1 and autoantibodies are a secondary, protective response aimed at clearing apoptotic cells (Mizoguchi, Mizoguchi, and Bhan 1999). Crossreactivity of peripheral-blood and colonic-lamina-propria CD4+ T cells with indigenous flora (bacteroides, bifidobacteria, and various enterobacteria) in patients with ulcerative colitis and Crohn's disease suggests that abnormal T-cell-specific immune responses to host flora are important in disease pathogenesis (Duchmann et al. 1999). Compared with circulating T cells, T cells in the lamina propria are more susceptible to Fas-mediated apoptosis(Boirivant et al. 1996)—a physiological process of cell death that, if altered, could contribute to inflammation. Furthermore, the Fas ligand (FasL) is strongly expressed by T cells in active ulcerative colitis but not in Crohn's disease lesions, suggesting that Fas-FasL induced apoptosis plays a part in mucosal damage of ulcerative colitis (Ueyema).

Although cytokine patterns are less clear in ulcerative colitis than Crohn's disease, in established colitis there seems to be a modified Th-2 response associated with upregulation of cytokines such as interleukin-5 and interleukin-10, but not interleukin-4 (Fuss et al. 1996). This disease was considered to have a T_H2 profile, but the concentrations of IL-4 and IL-5, which are normally elevated in T_H2 responses, have

been variable in ulcerative colitis tissues(Fuss et al. 1996). On the basis of results obtained from studies of the oxazalone colitis model, one of the few models to exhibit a T_H2 profile, Fuss and colleagues have suggested that ulcerative colitis has an atypical T_H2 response, mediated by natural killer T cells that secrete IL-13 (Fuss IJ 2004 JCI). These natural killer T cells are activated by APCs that express the nonclassical major histocompatibility complex (MHC) molecule, CD1d, which presents lipid rather than protein antigens to T cells. These unique observations could explain some of the discrepancies of past studies, but will require confirmation before being accepted. If true, blockade of IL-13 could provide an exciting new approach to ulcerative colitis treatment.

By contrast, mucosal cytokine and double-knock-out mouse studies have underscored the pivotal part played by interleukin-4 in the development of active colitis(Inoue et al. 1999; Mizoguchi et al. 2003). Results from knock-out mice have also shown that the immunoregulatory cytokines, interleukin-10 and tumour growth factor β, produced by the regulatory CD4+T cell subsets T-regulatory-1 (Tr-1) and Th-3, respectively, have pivotal roles in mediating tolerance towards luminal antigens(Liu et al. 2000; Groux et al. 1997). Furthermore, oral intake of antigen can induce tolerance and resolution of colitis by stimulating Th-3 regulatory cells specific for these antigens and thereby resetting the regulator-effector T-cell balance(Neurath et al. 1996), which has important implications for treatment and might account for the benefits of probiotics in clinical trials.

The gut mucosal immune system is in permanent contact with billions of commensal intestinal bacterial flora (10^{12} bacteria/g faeces in the colon, and consisting of 400 different species, with anaerobes predominating). Mucosal tolerance is an active process

by which an injurious immune response is prevented, suppressed or shifted to a non-injurious type of immune reaction. The most compelling evidence for the importance of gut microflora with the pathogenesis of ulcerative colitis comes from genetically engineered animals that develop colitis when exposed to non-pathogenic colonic bacterial microflora (in an environment free of specific pathogens), but not when they are in a sterile germ-free environment. Furthermore, experimental colitis is attenuated when animals are treated with broad spectrum antibiotics(Strober, Fuss, and Blumberg 2002). Enteric microflora can stimulate immune responses either by functioning as adjuvants or antigens. As adjuvants they activate innate immune responses, including dendritic cells and other APCs, and as antigens they stimulate the clonal expansion of T cells that selectively recognize the antigen through their T-cell receptor.

Numerous bacterial adjuvants, most notably lipopolysaccharide, peptidoglycan, flagellin and nonmethylated DNA (CpG motif), can bind selectively to various TLRs on innate immune cells, intestinal epithelial cells and mesenchymal cells (Cario, 2005 3189 /id). Ligation of these TLRs activates NFκB and the mitogen-activated protein kinases, which stimulate the transcription of a host of proinflammatory and regulatory genes. Activation of macrophages from susceptible individuals, by enteric lipopolysaccharide, peptidoglycan, flagellin or CpG, stimulates the production of IL-1β, TNF, IL-6, IL-8 and other chemokines, IL-12 p40 (and thus IL-12 and IL-23), adhesion molecules, IL-18, reactive oxygen species, nitric oxide and leukotrienes, which can all participate in the inflammatory response. In addition, NFκB activation of APCs (including dendritic cells) by microbial adjuvants induces the expression of MHC class II antigens, co-stimulatory

molecules, IL-12 and IL-23, which can activate TH1 and TH17 cells, respectively, if the appropriate antigen is present. Lipopolysaccharide can stimulate IL-12 p40 production by bone-marrow-derived dendritic cells in IL-10-deficient mice, and colonization of previously germ-free rodents with various commensal bacterial species can induce ICAM1 and IL-6 expression by intestinal epithelial cells. *In vivo* studies show that commensal bacteria selectively activate IL-12 p40 in dendritic cells of the distal ileum. Bacterial flagellin is both an antigen and adjuvant. A form of flagellin was shown to be a dominant antigen in experimental colitis and to induce serum antibody production in 50% of Crohn's disease patients(Lodes et al. 2004; Targan et al. 2005). Flagellin binds to TLR5 to activate NFκB. In addition to their proinflammatory properties, bacterial adjuvants can induce protective anti-inflammatory responses. For example, lipopolysaccharide stimulates IL-10 production in dendritic cells from normal mice, (Hoentjen Blood 105) and certain CpG preparations can prevent the onset of experimental colitis by inducing the production of type 1 IFN (IFN-α/β) in plasmacytoid dendritic cells, via TLR9 ligation(Katakura et al. 2005).

The role of the antigenic properties of bacteria in the pathogenesis of IBD is less clear. The concept of dysbiosis, where the altered balance of beneficial versus aggressive microbial species leading to a pro-inflammatory luminal milieu, resulting in a chronic inflammatory state in a susceptible host has recently received much attention. Numerous studies have implicated several commensal organisms, such as *E. coli*, *Bacteroides*, *Enterococcus* and *Klebsiella* species, in the pathogenesis of experimental intestinal inflammation and human IBD(Sartor 2006) By contrast, various *Lactobacillus* and

Bifidobacterium species have predominantly protective effects and have been used therapeutically as probiotics. Several groups have documented alterations in luminal or adherent microbial commensal flora in patients with Crohn's disease, ulcerative colitis and pouchitis(Swidsinski et al. 2002).

The high prevalence (about 70%) of pANCA in ulcerative colitis(Saxon et al. 1990) and even higher prevalence in patients with primary sclerosing cholangitis, refractory left-sided ulcerative colitis(Sandborn et al. 1996), and those who develop chronic pouchitis after ileal-pouch anastomosis(Sandborn et al. 1995) are the most reproducible data supporting non-epithelial autoimmunity. However, evidence that pANCA is a marker of genetic susceptibility to ulcerative colitis has been less convincing(Papo et al. 1996). These autoimmune proteins clearly occur more frequently in ulcerative colitis than Crohn's disease, but they have not been shown to cause tissue damage. Rather, they seem to be markers of underlying immune dysregulation or show crossreactivity with environmental factors, including commensal colonic bacteria.

1.4.2 The importance of gut barrier defence in ulcerative colitis

In the recent years, the importance of gut barrier defence in the aetiopathogenesis of IBD has been subject of considerable research interest and has been in part catalysed by genetic studies. The ABCB1/MDR1 (discussed in detail below), DLG5, OCTN1/2 and MYOB9 genes implicated in genetic susceptibility of IBD are all involved in the maintanence of epithelial integrity/defence. ABCB1/MDR1 and MYOB9 are both linked to the development of UC. The CARD15 gene might mediate intracellular bacterial clearance and also gut barrier defence by the production of antimicrobial α-defensins by Paneth cells.

The interface between luminal contents and intestinal epithelium constitutes the largest area of interaction between the host and environment. Critical to this interface is the ability to allow transcellular permeation of essential luminal molecules such as nutrients, but at the same time, to exclude or counter potentially harmful substances in the intestinal lumen. The cells of the mucosal immune system are protected from the large antigenic load in the gut lumen by a single layer of epithelial cells. Detoxification of xenobiotics, including toxins, carcinogens, and drugs, is the central task of many metabolising enzymes in the body. Detoxification and biotransformation of luminal agents is an important protective function of intestinal epithelial cells(Roediger and Babidge 1997). Several results from animal models of colitis as well as studies in human patients with IBD suggest that detoxification enzyme depletion may be an important event in the initiation and progression of UC and that excess biotransformation may result in colon cancer(Roediger and Babidge 1997). In animals, glutathione deficiency leads to enhanced

oxidative damage of mucosa cells and causes colitis(Martensson, Jain, and Meister 1990), whereas trinitrobenzene sulfonic acid– induced colitis can be significantly improved by glutathione supplementation(Loguercio C et al. 2003). In addition, mice with a combined deficiency of glutathione peroxidase 1 (Gpx1) and 2 (Gpx2) develop symptoms consistent with IBD(Esworthy et al. 2001; Esworthy et al. 2003) and low glutathione levels have been found in noninflamed as well as inflamed mucosa of patients with CD(Sido et al. 1998). Two groups of enzymes are known to handle metabolism of harmful compounds. The group of cytochrome P450 isoenzymes (CYPs) comprises numerous isoforms (approximately 60 are expected in humans) leading to oxidation (mostly hydroxylation) of molecules. Isoforms in the CYP groups 1, 2, and 3 mediate metabolism of many exogenous compounds. Many toxins and carcinogens require activation by CYP450 isoenzymes to obtain their reactive (that is, alkylating) properties in the body.

In general, three phases of detoxification processes can be defined. Phase 1 reactions include redox reactions via cytochrome P450 (CYP) enzymes(Ding and Kaminsky 2003), hydration by carbonic anhydrases(Fonti et al. 1998), and hydrolysis of compounds by carboxylesterases(Schwer et al. 1997). Phase 2 reactions comprise the sulfation, glucuronidation, acetylation, methylation, and glutathione conjugation of reactive intermediates leading to more hydrophilic molecules, which can be more easily exported from cells(Chen et al. 2003; Back and Rogers 1987; Pacifici et al. 1988). Most recently, the focus has been on the Phase 3 reactions- in the 'excretion' of xenobiotic compound back to the gut lumen(Dietrich, Geier, and Oude Elferink 2003).

Recent interests have focused on the class of proteins, the ABC efflux export transporters, which are highly expressed in the intestinal epithelium. These proteins/pumps reduce the local cellular burden of toxic compounds giving the individual cell protection against toxic effects. These transport proteins are primarily expressed in the apical membrane of epithelial cells, such as enterocytes, subjected to highest concentrations of exogenous xenobiotics. In these cells the same transporters function on the one hand to reduce the entrance of harmful substances and on the other hand to eliminate their detoxification products. The latter step has been called ''phase III metabolism'', indicating the close connection to the oxidation and conjugation steps of detoxification. The first function (that is, direct elimination of xenobiotics on entrance into the cell) represents a first defence line against xenobiotics and likewise could be called ''phase 0 metabolism''. It is pertinent that ABC proteins and CYP450 proteins co-localize in the intestinal epithelial cells. With a considerable overlap of an extensive list of substrates, there is a symbiotic relationship between these two classes of proteins in xenobiotic defence and metabolism(Benet and Cummins 2001).

P-glycoprotein 170 encoded by MDR1 gene is the archetypal efflux transporter protein, responsible in the the transport of a vast range of substrates. The role of this protein in disease pathogenesis and gut barrier defence is discussed in detail in the subsequent chapter.

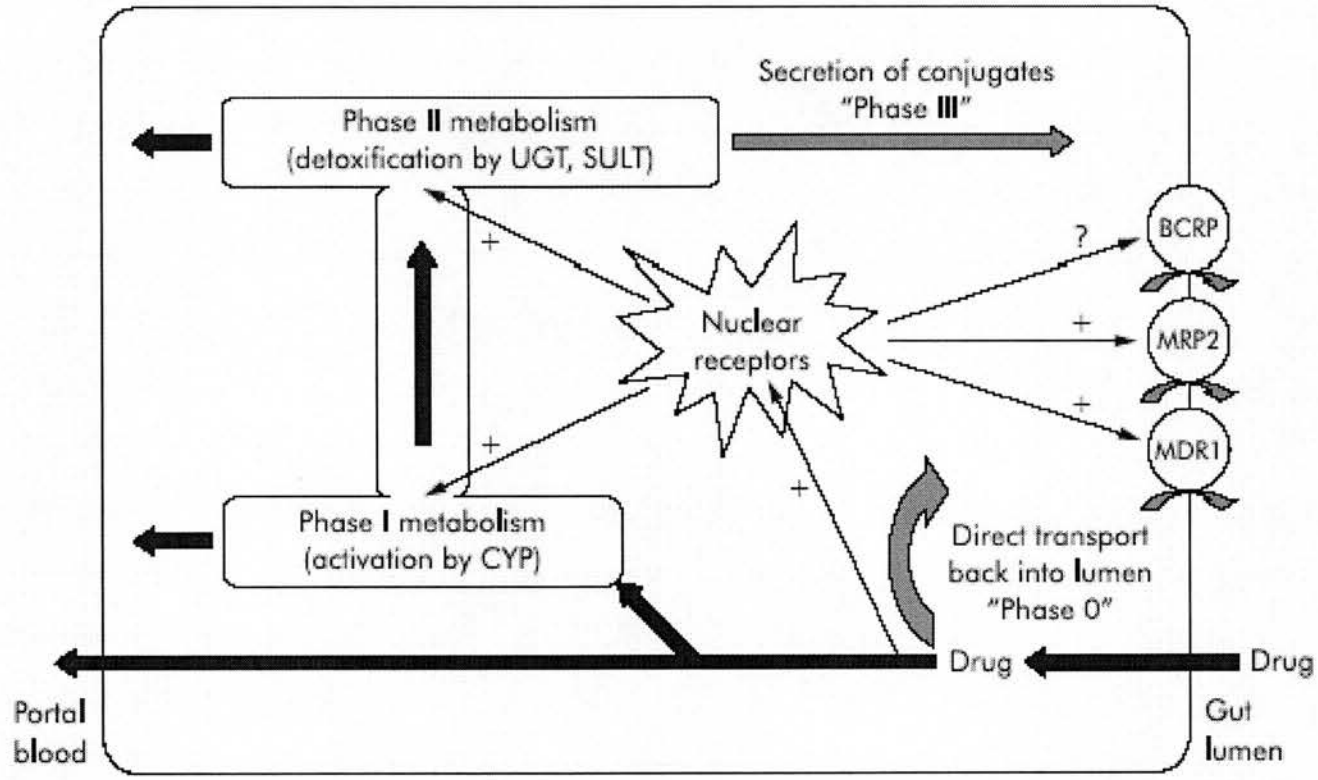


Figure 2: The schematic illustration of the importance of cellular transporters in gut barrier defence and detoxification.

1.4.2 Multidrug resistance gene (ABCB1/MDR1) gene: a key determinant of gastrointestinal disease.

The multidrug resistance pump (P-glycoprotein) is a 170 kDa phosphorylated and glycosylated plasma membrane protein belonging to the ATP-binding cassette superfamily of transport proteins encoded by the multidrug resistance genes (MDR), first described by Juliano and Ling in 1976(Juliano and Ling 1976). In man, two MDR genes, MDR1 and MDR3 (also called MDR2) have been described; in rodents, three – *mdr1a*, *mdr 1b* and *mdr2*(Hsu, Lothstein, and Horwitz 1989; Chen et al. 1986; Ueda et al. 1987). Only the MDR1 gene in humans and *mdr1b* and *mdr1a* genes in rodents, appear to be involved in drug transport and the development of drug resistance(Fardel et al. 2001). The human MDR3 and murine *mdr2* genes encodes a P-glycoprotein that does not seem to have a role in drug transport in intestinal epithelium, but has a role in the secretion of phosphatidylcholine into bile in the biliary tract(Smit et al. 1993). In humans, mutations in the MDR3 genes are linked to the development of progressive familial cholestasis(Deleuze et al. 1996; Jacquemin 2000).

P-glycoprotein is a transmembrane protein which is 1280 amino acid long and consists of two homologous halves of 610 amino acids joined by a flexible linker consisting of 60 amino acids. Each half has an N-terminal hydrophobic domain containing six transmembrane domains followed by a hydrophilic domain containing a nucleotide-binding site. The nucleotide binding sites can bind ATP and its analogues and both halves are essential since inactivation of either site inhibits substrate stimulated ATPase activity (Fig 3). However, the two sites are likely to be

functionally independent and cleavage probably occurs at one site at a time(Nagata et al. 2000).

In the human gastrointestinal tract, P-glycoprotein is found in high concentrations on the apical surfaces of superficial columnar epithelial cells of the colon and distal small bowel. High levels of P-glycoprotein are also found on the apical surfaces of epithelial cells in small biliary ductules, small ductules of the pancreas, proximal ductules of the kidneys and adrenal glands(Thiebaut et al. 1987). P-glycoprotein is richly expressed on the subapical surface of the epithelium of the choroid plexus of the brain (which forms the blood-CSF-barrier) as well as the luminal surface of the endothelium of the blood capillaries of the brain (blood-brain-barrier)(Wijnholds et al. 1997; Rao et al. 1999; Cordon-Cardo et al. 1989; Zhao et al. 1993). In the haemopoeitic system, peripheral blood mononuclear cells, macrophages, natural killer, dendritic cells, T and B-lymphocytes all express P-glycoprotein at varying levels(Klimecki et al. 1994). In the gastrointestinal tract, there is a regional variation in P-glycoprotein expression. Various methodologies have been used to investigate this (Table 1). Within the small intestine, there is clear evidence demonstrating that P-glycoprotein is maximally expressed in the epithelial cells of the ileum with a gradual decrease proximally into the jejunum, duodenum and stomach. Variations of P-glycoprotein expression across the length of the colon are less well-defined (Chianale et al. 1995).

The role of P-glycoprotein: Host-bacterial interactions

The available literature suggests that P-glycoprotein act as a transmembrane efflux pump, which removes drugs from the cell membrane and cytoplasm. However, the normal physiological function of P-glycoprotein in the healthy gastrointestinal tract remains under investigation and may be complex. Expression of P-glycoprotein on the luminal surfaces of the epithelial cells of the small and large intestines, biliary ductules and proximal tubules of the kidneys suggest a role in decreasing (Yumoto et al. 2001; Fricker et al. 1996; Fojo et al. 1987)the absorption from the gut and secretion of endogenous and exogenous hydrophobic amphipathic toxins(Trezise et al. 1992).

Perhaps the greatest insight into the physiological importance of P-glycoprotein in the gastrointestinal tract has from the description of the phenotype of genetically engineered mice lacking the *mdr1a* gene. The investigators had been stimulated to develop this model by the finding that the MDR1 gene is present in a region of the human genome (7q21.1) that may harbour a disease gene involved in susceptibility to inflammatory bowel disease (Satsangi et al. 1996b). Panwala et al. demonstrated that spontaneous colitis developed in these *mdr1a* knockout mice when maintained under specific pathogen free conditions (Panwala, Jones, and Viney 1998). This was reversed and prevented by the administration of antibiotics, suggesting that intestinal flora was necessary in the initiation and perpetuation of colitis in this model. Other studies have confirmed that these mice have normal viability, fertility, immunology and range of biochemical parameters (Schinkel et al. 1997; Wijnholds et al. 1997). Together with the protective effect of antibiotics, these observations imply that the

loss of xenobiotic efflux mechanism underlies the development of colitis in this model. Bone marrow transfer studies involving the wild type donor cells into mdr1a deficient recipients demonstrated that the colitis develops in these mice because of the mdr1a deficiency in the epithelial cells rather than in lymphoid or myeloid cells. The colitis associated with *mdr1a* deficiency has been regarded as a model of ulcerative colitis with the presence of long dysregulated crypts, crypt abscesses and superficial mucosal inflammation. However, transmural infiltration of B and T-cells was also observed, not dissimilar to the histological appearances of Crohn's disease.

The susceptibility to the development of colitis increased as the mice *(mdr1a -/-)* aged (Panwala, Jones, and Viney 1998). The gradual acquisition of commensal luminal organisms in the background of defective efflux capacity of epithelial cells may be the reason although clearly other effects of ageing maybe pertinent. In addition, a recent study has added further intriguing insight into the interaction between P-glycoprotein and the gut flora. In *mdr1a* -/- mice, infection with *helicobacter bilis*, accelerated the development of colitis while *helicobacter hepaticus* infection delayed the development of spontaneous disease. Helicobacter-induced IBD in *mdr1a* -/- mice maybe mediated via a family of bacterial toxins (eg. cytolethal distending toxins) which are virulence factors in certain helicobacter infections. However, it is important to emphasise that these mice developed spontaneous colitis independent of helicobacter infection. The mechanisms for these findings suggest that different luminal bacteria may influence the development of colitis and its progression. Both helicobacter bilis and hepaticus have been shown to induce colitis in mice lacking T and B cells (SCID mice)(Shomer et al. 1998; Cahill et al. 1997; Burich et al. 2001; Cahill et al. 1997). Recently, other models such as dextran-sulphate induced colitis;

TCRα knockout and CD45RB colitis have all been characterised by reduced expression of intestinal mdr1a in the presence of inflammation(Iizasa et al. 2003; Mizoguchi et al. 2003). In support of this, data from our unit have specifically showed that the PgP expression is significantly lower in the HLA B27 transgenic models compared with non-transgenic mice(Moodie et al. 2004), of the same background. Expression in this model is again affected critically by germ-free conditions.

It is of great interest to note that PgP function has now been linked to the development of colorectal carcinoma in mdr-1a deficient mice. Maggio-Price et al. demonstrated that a proportion of dual-infected mdr1a(-/-) with *Helicobacter bilis* and *hepaticus* mice showed IBD with foci of low- to high-grade dysplasia(Maggio-Price et al. 2005). A group of dual-infected mdr1a(-/-) animals which were maintained long term by intermittent feeding of medicated wafers to model chronic and relapsing disease showed a higher frequency of high-grade crypt dysplasia, including invasive adenocarcinoma. Colonic epithelial preparations from co-infected mdr1a (-/-) mice showed increased expression of c-myc (5- to 12-fold) and interleukin-1alpha/beta (600-fold) by real-time polymerase chain reaction relative to uninfected wild-type and mdr1a (-/-) animals. It remains speculative but the circumstantial evidence based on the mdr-1a mice has a number of similarities to the clinical course of human UC.

These findings support the hypothesis that *mdr1a* deficient mice developed mucosal inflammation not because of increased permeability but as a result of an increase of bacterial activation of the epithelial cells. This effect might be pro or anti-inflammatory and again, the identity of the bacterium involved may be the critical determinant. It has been demonstrated that bacterial flagellin signalling through the

TLR5 receptor can lead to increased NFκB expression in epithelial cells (Gewirtz et al. 2001). On the other hand, Neish et al demonstrated that certain non-pathological organisms can lead to a block in the ubiquitination of IκBa (necessary for IκBa degradation) and thus a subsequent block in NFKB translocation to the nucleus (Neish et al. 2000). In either of these cases, if these events are caused by bacteria entering the epithelial cells, it is possible to envisage how the function of the epithelial cells can be influenced by a defective efflux transporter and the bacterial contents of the lumen.

Pharmacokinetic effects

The effect of P-glycoprotein on the pharmacokinetics of drugs has been well demonstrated in two studies (Sparreboom et al. 1997; Mayer et al. 1996). In *mdr1a-/-* mice, the systemic bioavailability for the substrates, digoxin and paclitaxel was markedly increased compared with wild-type mice following oral administration. Similarly, faecal levels of these drugs were reduced following intravenous administration. The range of substrates transported by P-glycoprotein is enormous and includes a variety of structurally and pharmacologically distinct hydrophobic compounds (Table 2). There is accumulating evidence that interactive alliances also exist between xenobiotic metabolising enzymes and P-glycoprotein. The best-defined alliance is between cytochrome P450 3A (CYP3A) and P-glycoprotein. These proteins co-localise in the small intestinal epithelial cells and have a considerable overlap in substrate specificities (Benet and Cummins 2001). As P-glycoprotein can influence the intracellular concentrations of many CYP3A substrates, it may also affect the availability of those substrates to CYP3A and thereby, the extent of CYP3A metabolism of those substrates.

1.4.4 Regulation of P-glycoprotein

The transcriptional regulation of MDR-1 gene is unexpectedly complex and far from being understood. The transcriptional regulators of the human multidrug resistance gene are discussed in detail in the review by Labialle et al (Labialle et al. 2002). P-glycoprotein dependent drug transport activity depends on the level of expression of the MDR1 gene as well as on the functionality of the MDR-1 encoded P-glycoprotein. The level of intestinal P-glycoprotein shows wide interindividual differences controlled by both environment and genetic factors.

Pharmacological regulation of P-glycoprotein

Shapiro et al. showed that P-glycoprotein mediated drug transport was stimulated by the antihypertensive drug, prazosin, and the hormone, progesterone (Shapiro et al. 1999). Rifampicin also has been shown to induce P-glycoprotein expression and underlies the mechanism responsible for reduced digoxin levels during concomitant therapy (Greiner et al. 1999). In healthy male volunteers, the oral bioavailability of digoxin decreased by 30% and the intestinal P-glycoprotein levels were induced 3.5 fold during rifampicin therapy. Induction appears to take place at transcriptional level, as MDR1 mRNA levels are elevated as well after treatment with inducer drugs (Asghar et al. 2002) (Westphal et al. 2000). It has been suggested that the induction of the MDR1 is mediated by the pregnane X receptor (which is also responsible for the induction of CYP3A) which binds to PXR response elements situated upstream in the MDR1 gene (Geick, Eichelbaum, and Burk 2001).

The role of corticosteroids on the expression of P-glycoprotein has been an area of considerable interest as a possible mechanism for corticosteroid resistance. The high

levels of expression in the adrenal glands and endometrium suggest a role in the transport of steroid-related compounds. In addition, these compounds are all substrates of P-glycoprotein. Nevertheless, the available literature is conflicting and complex (Table 3). In mouse hepatoma cell cultures treated with dexamethasone, Zhao et al. demonstrated induction of mdr1 and mdr3, but not mdr2 transcripts. Similarly, MDR1 mRNA levels were elevated in dexamethasone treated human hepatoma line. This effect was not seen in non-hepatoma cell line (HeLa)(Zhao et al. 1993). In another study examining the expression of CYP3A and mdr in rat liver, dexamethasone was found to induce mdr2 expression only in male livers. Curiously, mdr1a and 1b were not induced in either sex (Salphati and Benet 1998). Lin et al showed that dexamethasone induces P-glycoprotein in the intestines and liver of rats with the functional effect of a decreased plasma substrate level (indinavir) (Lin et al. 1999). However, Demuele et al. found no increase in the intestinal expression. In these animals, the expression was upregulated in the lungs and liver where as an opposite effect was observed in kidney tissue (Demeule et al. 1999).

Although P-glycoprotein expression maybe influenced by corticosteroids or other pharmacological agents, recent data illustrates that the level of expression may not necessarily correlate with its function. Murakami et al. demonstrated that although, increased P-glycoprotein expression was evident in rats with induced acute renal and liver failure, the *in vivo* P-glycoprotein activity was significantly depressed. The plasma from these diseased rats (with renal and liver failure) demonstrated a significantly greater inhibitory effect on P-glycoprotein function (in Caco-2 cell line) compared with dexamethasone treated-rats (Murakami et al. 2002). It is hypothesised

that in disease states, endogenous related compounds such as corticosterone (a P-glycoprotein inhibitor) may participate to suppress the *in vivo* function.

Another intriguing aspect of the pharmacological regulation of the expression of P-glycoprotein is seen in the multidrug resistance phenomenon. This is observed in tumour cells which, following initial contact with on one anticancer agent, become rapidly resistant to other unrelated agents. This phenotype is mainly due to the over expression of the MDR-1 gene. One possible mechanism for this may be through gene rearrangement. Mickley et al. found that in several drug resistant cancer cell lines as well as samples from two leukaemic patients who had developed drug resistance, gene rearrangement had occurred resulting in the activation or increased expression of MDR1(Mickley et al. 1997). Although a feasible hypothesis, there is no evidence to support selection of aberrant clones of the MDR gene as the cause of the development of multi-drug resistance(Van Den Heuvel-Eibrink et al. 2001; Bouma et al. 1996a). The above studies suggest that regulation of the MDR1 gene expression is likely to be gene, cell and perhaps gender specific. Furthermore, future work in this area will have to consider not only the expression but also the function of P-glycoprotein as well.

Genetic influences on P-glycoprotein function and expression

The MDR1 gene lies on chromosome 7q21.1. Several polymorphisms of the gene have been characterised (Table 4) (Cascorbi et al. 2001b). Hoffmeyer et al. first described a single base polymorphism in exon 26 (C3435T) and suggest that this variant influences P-glycoprotein expression in the duodenum and P-glycoprotein activity(Hoffmeyer et al. 2000a). In this study, healthy individuals homozygous for this variant (TT genotype) has significantly lower duodenal expression of P-

glycoprotein and higher serum digoxin levels. Moreover, evaluation of maximum plasma concentrations during steady state of digoxin administration showed statistically significant mean increase of 38% in the homozygous TT genotype compared with the CC genotype.

This variant has received considerable attention. However, controversy still exists regarding the functional effect of this polymorphism and the current literature remains rather contradictory. It is particularly noteworthy that the MDR1 C3435T allelic variant, a single nucleotide polymorphism, is non-coding and does not affect amino acid sequence change. Although a number of coding mutations within the MDR-1 gene (Table 4) have been identified, none of this variants are in strong linkage disequilibrium with the C3435T variant(Cascorbi et al. 2001b; Drescher et al. 2002). The extent of linkage disequilibrium also depends strongly on ethnicity (Tang et al. 2002). Kimchi-Sarfaty et al. demonstrated that there were no functional consequences of the five commoner amino-acid altering single nucleotide polymorphisms (including 2677 SNP) in this gene, on both cellular localisation and transport function of P-glycoprotein (Kimchi-Sarfaty, Gribar, and Gottesman 2002). The observed functional effect ascribed to the C3435T variant may reflect linkage disequilibrium with a polymorphism elsewhere in the genome that modifies MDR1 expression and function. However, at present this hypothesis requires further detailed studies of the gene.

Overall, the available evidence suggests that the C3435T polymorphism is predictive of MDR-1 expression. Kim et al showed that MDR 1*2 haplotype (G2677T/C3435T) was associated with increased P-glycoprotein function in vitro and low concentrations of a known substrate, fexofenadine(Kim et al. 2001a). Fellay and colleagues,

demonstrated that the MDR1 3445 TT genotype was associated with a low expression of MDR1 transcripts and P-glycoprotein expression in peripheral blood mononuclear cells with low plasma levels of nelfinavir and efavirenz (antiretroviral agents)(Fellay et al. 2002). Hitzl and colleagues showed that in CD56+ cells (NK cells), MDR1 3445 TT had lower P-glycoprotein function and expression compared with the CC genotype (Hitzl et al. 2001). In another study, Nakamura et al. investigated the effect of the C3445T mutation on the expression level of MDR1 mRNA in duodenal enterocytes of healthy Japanese subjects (Nakamura et al. 2002) and found significantly elevated levels of transcripts in those patients with TT compared with CC allele. Given the likely complicated interactions with cytochrome P450 system and the presence of other poorly characterised transporter systems, the relationship between the function of P-glycoprotein and drug substrate level is debatable. This consideration and the differences in study designs may explain these apparently contradictory results. Moreover, there maybe cell specific differences in regulation. There is a clear need for further well-designed studies using *in vivo* and *ex vivo* systems to reconcile these differences.

The model of the involvement of PgP in the aetiopathogenesis of IBD that logically follows from consideration of the phenotype of the mdr1a (-/-) model is that low intestinal PgP expression may predispose to inflammation, and high levels of expression may protect. Thus genetic influences that determine the expression and functionality of PgP may be expected to influence the susceptibility to IBD (assuming that PgP plays a pivotal role in pathogenesis). It is noteworthy that the MDR1 gene maps to chromosome 7q22, a locus of IBD susceptibility as identified by genome wide scanning (Satsangi et al. 1996b). The MDR1 gene is composed of 28 exons and

is 209 kb in length, and more than 30 SNPs have been described(Hoffmeyer et al. 2000a; Cascorbi et al. 2001a; Kim et al. 2001b).

The two previously described SNPs, exonic variant C3435T and G2677T/A have been shown to correlate with activity/expression of P-glycoprotein 170 and have received most attention. The C3435T SNP in exon 26 has been most extensively investigated and was first shown to correlate with expression of PgP(Hoffmeyer et al. 2000a). In this study, the TT genotype was associated with decreased intestinal P-glycoprotein 170 expression with functional consequence, as inferred from measurements of the systemic bioavailability of digoxin , a PgP substrate, following oral administration (due to decreased excretion of digoxin by PgP). This has been replicated in other pharmacokinetic studies(Hitzl et al. 2001; Kim et al. 2001a; Johne et al. 2002b). In addition, the effect of the MDR1 C3435T SNP and its postulated correlation with P-glycoprotein 170 activity/expression has been shown to play a role in drug-resistant epilepsy(Siddiqui et al. 2003), immune recovery after initiation of anti-retroviral therapy in HIV(Fellay et al. 2002) and the development of renal cell carcinoma(Siegsmund et al. 2002).

1.4.5 The role of ABCB1/MDR1 gene in pharmacogenetics

The multidrug resistant gene has been postulated to contribute to the phenomenon of corticosteroid resistance in inflammatory bowel disease. Farrell and colleagues, demonstrated that P-glycoprotein 170 expression in peripheral blood lymphocytes was associated with failure of medical treatment in IBD(Farrell et al. 2000). The authors also demonstrated that PgP expression appeared to be constitutive as levels were similar when active IBD patients were followed up 3 months later; and suggested that

genetic factors may play an important role in determining corticosteroid resistance in IBD. Farrell and his colleagues investigated the role of P-glycoprotein on lymphocytes as a factor in determining responsiveness to medical therapy (Farrell et al. 2000). Corticosteroids and cyclosporin are both substrates of P-glycoprotein (Ueda et al. 1992). Peripheral blood lymphocytes in patients with active Crohn's disease (15 patients) and ulcerative colitis (28 patients) in whom medical therapy had failed and surgical intervention had been necessary, were shown to have a higher expression of P-glycoprotein compared with those who had inactive disease and required surgery for obstruction (25 patients) and dyplasia (13 patients) respectively ($p=<0.0001$ and 0.0002 respectively). Interestingly, in ulcerative colitis, there was no significant difference when P-glycoprotein expression was compared between the patients requiring colectomy for failed medical treatment and the group with active UC ($p=0.6$). The intraepithelial lymphocyte expression of P-glycoprotein was shown to correlate significantly with peripheral blood lymphocyte expression. Farrell et al. went on to demonstrate that by blocking the action of P-glycoprotein on human cell lines, higher intracellular levels of cyclosporin and corticosteroids were achieved (Farrell et al. 2002) and speculated that this may be a potential target for pharmacotherapy in a later study.

The functional consequences of this increased MDR1 expression and the effects of genetic polymorphisms within the MDR-1 gene on expression and function are important questions generated by this study. Perhaps most crucially, there is a need to elucidate whether there is a correlation between lymphocyte expression of MDR and the expression on intestinal epithelial cells in both the healthy gut and disease states.

With respect to MDR1 over-expression as a factor in corticosteroid-resistance, this has been studied in other conditions. In patients with glucocorticoid resistant asthma, P-glycoprotein expression on peripheral blood lymphocytes was not found to be increased compared with those with responsive or mild asthma (Montano et al. 1996). In patients with rheumatoid arthritis treated with glucocorticoids, the expression of P-glycoprotein on peripheral blood lymphocytes was increased compared with those without therapy (Maillefert et al. 1996). Patients with rheumatoid arthritis without corticosteroid therapy did not demonstrate a higher expression of MDR1 and this again may reflect a secondary effect to corticosteroids. Farrell et al. did not find any difference in the expression of P-glycoprotein in patients with active disease inflammatory bowel disease (80% on corticosteroids) compared with those with inactive disease (20% on corticosteroids)(Farrell et al. 2000). There are several caveats to these studies. Firstly, peripheral blood lymphocytes consist of different subsets of lymphocytes which express MDR-1 at varying levels (Ludescher et al. 1998). Indeed within the gastrointestinal tract, it is accepted that the lymphocytes homing to the gut-associated mucosal lymphoid tissue are phenotypically distinct to the circulatory lymphocytes. In addition, Yacyshyn et al, has demonstrated that the intra-epithelial, lamina propria and peripheral blood lymphocytes all demonstrated different levels of MDR-1 expression and activity in UC, CD and healthy controls. CD3 and CD8 lymphocytes also were present in different proportions in these groups of subjects. Furthermore, these subsets have intrinsically different levels of P-glycoprotein function (CD8 has higher levels of P-glycoprotein). Overall, MDR-1 expression and function were found by these investigators to be lower in UC compared with CD and healthy controls (Yacyshyn, Maksymowych, and Bowen-Yacyshyn 1999). Secondly, the effect of corticosteroids (or indeed other treatments

used in inflammatory bowel disease) on MDR-1 expression is not fully established. It remains to be seen whether higher expression of P-glycoprotein reflects a secondary phenomenon to corticosteroid therapy or a primary one, influencing the response to treatment. Finally, as discussed earlier, increased expression does not necessarily imply increased function in diseased states.

1.4.6 The role of Pregnane X-Receptor: Does defective detoxification impair barrier defence in IBD?

The importance of defective detoxification mechanisms coordinated by Pregnane-X receptor at the epithelial celullar level in IBD has recently been demonstrated (Langmann et al. 2004). PXR is a nuclear hormone receptor that is ligand-activated by a large number of structurally and pharmacological diverse endogenous compounds (including pregnanes, corticosteroids, rifampicin and bile acids)(Staudinger et al. 2001; Chrencik et al. 2005; Schuetz et al. 2001; Willson and Kliewer 2002). It is of interest to note that the function and expression of MDR1 is closely regulated by PXR(Kliewer, Goodwin, and Willson 2002). Central to the recent human microarray data Langmann et al., is the suggestion that PXR may also act in conjunction with MDR1 in determining susceptibility to IBD (in particular UC). Dysregulated PXR expression and function leading to the resultant widespread effect on the downstream detoxification genes therefore predisposes to increased risk of inflammation. Preliminary data (in abstract form) by Blokzijl et al. showed that PXR expression is indeed reduced in IBD, but this was also the case in non-IBD inflammatory tissues (such as collagenous colitis and diverticulitis)(H.Blokzijl 2005). Further detailed functional studies are clearly required to investigate this hypothesis. As highlighted earlier, these findings now also suggest that PXR itself may represent an attractive

candidate gene for IBD. In Langman's study, DNA microarray analysis involving non-affected colonic tissue of CD and UC, revealed a cluster of strongly down regulated detoxification genes (of the glutathione and sulfo-transferase family) and ABC transporters (including MDR1) in UC, together with the near complete loss of the transcriptional regulator pregnane X receptor (PXR). Strikingly also, the expressions of MDR1 and PXR were both reduced in tissues of patients with UC in subsequent rt-PCR analysis in the same study. PXR regulates the induction of many genes including CYP3A4 and the ABC transporter family genes (which includes MDR1). It is of interest to note that two earlier separate DNA microarray studies also showed downregulation of the MDR1 gene along with several other detoxification genes studied (carboxylesterase 2, oestrogen SULT and CYP1A1).

Therefore, the current available data suggest that efflux transport mechanisms mediated by ABC-transporters (and others such organic cation transporters) and also detoxification mechanisms at the epithelial interphase, play critical roles in barrier defence. Their respective precise mechanistic roles and cross-talk between the innate/adaptive immunity and inflammation remain unknown but represent important scientific questions. Several hypotheses are possible: a defect in mucosal barrier defence could lead to increased uptake of luminal antigens and/or adjuvants that overwhelm the net suppressive tone of the mucosal immune system. Alternatively, a defect in epithelial repair could potentiate damage by environmental triggers such as NSAIDs or infections that cause only transient damage in normal hosts. The net effect of either pathway is constant stimulation of innate and acquired mucosal immune responses by luminal adjuvants and antigens, respectively.

Table 4: Regional variation in P-glycoprotein expression in humans and rodents

Authors	Subjects	Methods	Findings
Yumoto et al (2001)	Mice	Efflux rate via rhodamine-123 assays	High in ileum compared with duodenum
Fojo (1987)	Human tissues	MDR1 mRNA	Colon > Jejunum > Rectum > Stomach/Oesophagus
Fricker (1996)	Human GI tract	MDR1 mRNA	Colon > Jejunum/Ileum > Stomach
Chianale (1995)	Rat intestine	mdr3 mRNA and rhodamine-123 assay	Ileum > Jejunum > Duodenum
Treziche (1992)	Rat intestine	mdr3 mRNA	Moderate expression in duodenum/jejunum, maximal expression in ileum and decrease expression from proximal to distal colon.

Table 5: List of P-glycoprotein substrates

Substrates
Steroid compounds
Aldosterone
Progesterone
Hydrocortisone
Cortisol
Corticosterone
Dexamethasone
Anticancer agent
Doxorubicin
Daunorubicin
Vinca alkaloids
Actinomycin D
Epotoside
Immunosuppressive agents
Cyclosporin
FK 506
Methotrexate
Protease inhibitors
Indinavir
Nelfinavir
Ritonavir
Antibiotics
Erythromycin
Rifampicin
Cardiac drugs
Digoxin
Quinidine
Lovastatin
Antihistamines
Terfenadine
Others
Domperidone
Loperamide

Table 6: Studies in the effects of corticosteroids on MDR/P-glycoprotein 170 expression. ↑ - *increased,* ↔ - *no change,* ↓ - *decreased, P-gp – P-glycoprotein 170.*

Authors	*Subjects*	*Dexamethasone treated*	*Effects*
Zhao et al. (1993)	Mouse hepatoma cell line	Yes	↑mdr1 and mdr3, no change in mdr2
	Human hepatoma cell line	Yes	↑MDR1
	HeLa cell line	Yes	↔ MDR1
Salpati et al (1998).	Rat liver	Yes	↑mdr2 only in male rats. ↔ mdr1 and mdr 3 in either sex.
Lin et al. (1999)	Rat liver and intestines	Yes	↑ P-gp both liver and intestines.
Demuele et al. (1999)	Rat organs	Yes	↑ P-gp in lungs and liver, ↓in kidneys, ↔in intestines
Murakami et al. (2002)	Rat intestines	Yes	↑ P-gp expression and function
	Rat intestines (induced acute liver and renal failure).	No	↑ P-gp expression but ↓function

Table 7: Cell populations and transporter substances in studies of MDR1 3445TT genotype.

Study	Cell type	Expression/function MDR1 3445T	Plasma drug concentrations
Hoffmeyer et al	Enterocytes	↓MDR1 mRNA	↑Digoxin
Fellay et al.	Peripheral blood mononuclear cells	↓MDR1 mRNA and Pgp expression	↓Nelfinavir and efinarvir
Kim et al	Transfected murine NIH-3T3	↑Pgp function in vitro for MDR1*2 haplotype	↓Fexofenadine
Hitzl et al.	CD 56 natural killer cells	↓MDR1 mRRNA and Pgp expression	NA
Nakamura et al.	Duodenal enterocytes	↑MDR1 mRNA	↓digoxin

Pgp – P-glycoprotein; MDR1*2 haplotype – G677T/C3445T; NA – not available, MDR – multidrug resistance, mRNA – messenger RNA, ↓= decrease and ↑=increase.

Table 8. Single nucleotide polymorphisms (SNPs) in the MDR1 gene.

SNP	Region	n	Heterozygous (%)	Homozygous (%)	Estimated	Effect
T-12C	Exon 1	85	11.8	0	0.4	Non-coding
G-1A	Exon 2	188	11.2	0	0.4	Translation
A61G	Exon 2	188	17.6	0.5	0.81	Asn21Asp
G-25T	Intron 4	85	26.0	3.5	2.3	Unknown
G-35C	Intron 4	85	1.2	0	0.01	Unknown
T307C	Exon 5	85	1.2	0	0.01	Phe103Leu
C+139T	Intron 5	85	48.2	16.5	16.8	Unknown
C+145T	Intron 5	85	2.4	0	0.01	Unknown
G1199A	Exon 11	85	12.9	0	0.4	Ser400Asn
C1236T	Exon 12	188	48.9	13.3	14.4	Gly412Gly
C+44T	Intron 12	188	11.7	0	0.4	Unknown
T-76A	Intron 16	85	45.9	22.4	20.3	Unknown
A+137G	Intron 17	85	1.2	0	0.01	Unknown
G2677T	Exon 21	83	43.4	42.2	38.4	Ala893Ser
G2995A	Exon 24	36	11.1	0		Ala999Thr
C3445T	Exon 26	537	47.7	26.4	24.1	Ile1145Ile
C3396T	Exon 26	188	0.53	0	0.01	Wobble

The positions of identified SNPs correspond to positions of the MDR1 cDNA (gb : M14758, codon TTC in exon 10) with the first base of the ATG start codon set to 1. SNPs that are located in introns are presented as exon +/-n, where n = nucleotide upstream (+) or downstream (-) of exons. The predicted ratios of homozygous genotypes (q2) were calculated on the basis of the Hardy-Weinberg distribution.

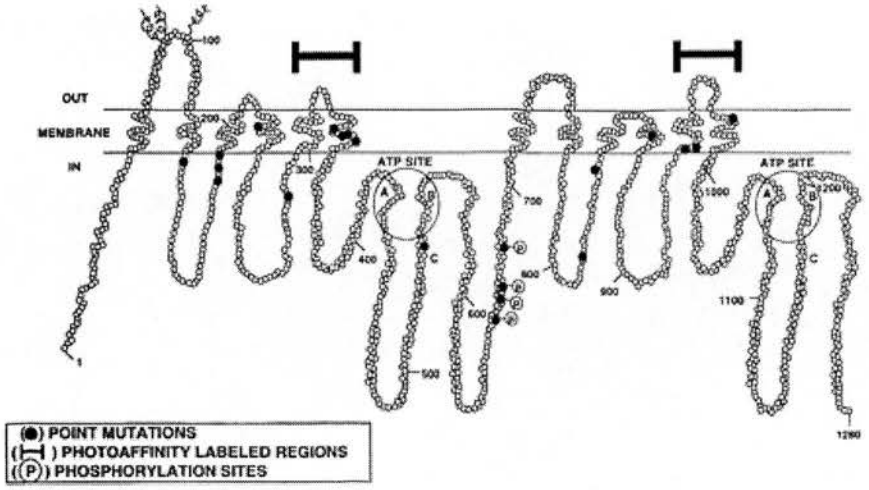


Fig 3: 2-Dimensional hypothetical structure of P-glycoprotein 170 pump.

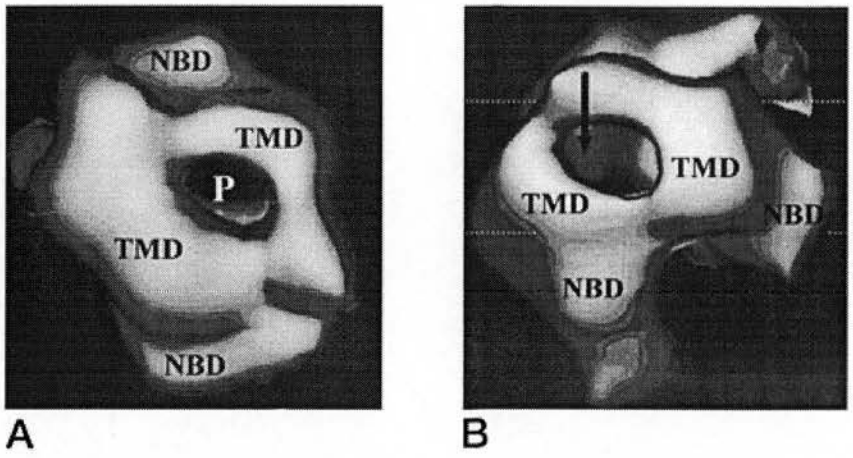


Fig 4.: 3-Dimensional hypothetical structure of P-glycoprotein (NBD – nuclear binding domain, P – pore, TMD – transmembrane domain, A- view from above, B – view from below.

1.5 Rationale and plan of thesis

The overall aims of this thesis are to investigate the roles of clinical and genetic factors in determining the course of ulcerative colitis. The rest of the thesis will describe the rational approach adopted, starting with clinical studies, followed by genetic association studies based on the highly accurate phenotypic data assimilated from the former. Chapter 2 will describe the overall material and methods employed in this thesis. Each of the subsequent chapters will start with a short introduction and more detailed description of methods pertinent to the respesctive chapter.

In chapter 3, the importance of corticosteroid dependence and resistance is examined in the first United Kingdom dataset in inflammatory bowel disease and the only the second study worldwide in ulcerative colitis. Detailed clinical parameters which may identify patients at increased likelihood of early non-response within 30 days of therapy and surgery within 1-year of follow-up are investigated. The current data highlighted the consistent nature of corticosteroid dependence/resistance and the need to understand the molecular mechanisms underlying this phenomenon.

In chapter 4, the development of a predictive model, which aids the selection of patients for early second-line medical therapy or surgery in severe ulcerative colitis, is presented. This study is based on the largest cohort of patients with unequivocal severe disease as defined by the modified Truelove and Witts criteria. A simple numerical index involving the stool frequency within 1st 3 days of intravenous corticosteroid therapy, serum albumin and radiological finding of colonic dilatation defines 3 risk groups: low, intermediate and high (10%, 45% and 85% risk of colectomy) is formulated and discussed.

In chapter 5, the association study involving 2 candidate polymorphisms (C3435T and G2677T/A) within the MDR1 gene in a Scottish-Caucasian cohort of 335 UC, 268 CD and 370 healthy controls is described. The overall association with T-allele and TT-genotype of the MDR1 3435 SNP is shown with UC. In addition, the novel associations with extensive UC (from detailed genotype-phenotype analysis) confirming the importance of phenotypic heterogeneity in association studies in IBD; and 2-locus haplotype analysis which demonstrated protective and susceptible haplotypes are discussed in depth. The latter has led to the generation of the hypothesis that genetic contribution may influence the functionality or expression of the gene product, P-glycoprotein 17o which in turn influences susceptibility to UC in a bi-directional fashion. The pharmacogenetic contributions of these polymorphisms in IBD are also investigated.

In chapter 6, the successful application of a gene-wide haplotype tagging strategy is shown using the MDR1 gene in assessing the overall contribution of the gene and to discriminate critical variants associated with disease susceptibility. The gene was re-sequenced in 24 CEPH trios and the haplotypic structure was determined. By using a haplotype r^2 method, the association of MDR1 gene was demonstrated in UC (and the phenotype of extensive disease). This association is shown to be critically dependent on an upstream intronic variant, rs3789243 and to be additive to other selected tagging SNPs.

In chapter 7, the application of candidate gene-wide tagging strategy is extended to investigate the contribution of the Pregnane-X Receptor gene to disease susceptibility

in IBD. Pregnane-X receptor is a transcriptional regulator of xenobiotic metabolizing enzymes and efflux transporters from the ATP-binding cassette, ABC-superfamily. Allelic variations of this gene have recently been shown to be associated with inflammatory bowel disease in an Irish dataset. The data generated is used to re-examine these associations within the Scottish dataset.

In chapter 8, we presented further genetic association of mutiresistant protein 3 ABCC3/MRP3) with inflammatory bowel disease. Haplotypic analyses revealed significant associations in independent Scottish and Swedish case-control cohorts. In addition, we have presented detailed haplotypic-phenotypic subanalyses and also stratification with ABCB1/MDR1 and PXR genotypes to determine gene-gene and gene-environmental interactions with ABCC3/MRP3 gene.

In chapter 9, the implications and future directions of the data presented in chapters 2-8 are discussed.

Chapter 2

Materials and Methods

2.1 Patients

1.1.1 Definition of ulcerative colitis and Crohn's disease

The definitions of ulcerative colitis and Crohn's disease were based on the classifications as described by Lennard-Jones(Lennard-Jones 1989). This original classification set out a list of exclusion criteria (infection, ischaemia for example) and; histological and clinical features associated with each respective entity. There was ratification by an experienced histopathologist for each diagnosis, and patients with indeterminate disease, were excluded. Patients with acute severe UC were defined according to the modified Truelove and Witts criteria (Presence >6 bloody stoos with one or more the following features: tachycardia, fever, anaemia or elevated ESR).

2.1.2 Recruitment of patients and healthy controls

3.1.3 Patients were recruited from the gastroenterology units of Western General Hospital, Royal Infirmary Edinburgh and St. John's Hospital, Livingston (all in Lothian region serving a catchment population of approximately 500 000 patients) between 1997-2003. In the genetic case-control studies, controls were actively recruited from healthy volunteers and also obtained from anonymous Scottish-Caucasian blood donors from the Lothian region. The demographic details and numbers used are described in detail in the subsequent chapters (4,5,6,7 and 8). The number of IBD patients varied between chapters, due to differences in study recruitment phases and designs. In chapter 3, the IBD cohort comprised of newly diagnosed patients commenced on corticosteroids; in chapter 4, the cohorts were recruited with acute severe UC (satisfying Truelove and Witts criteria). In chapters 5 to 8, the increasing number of IBD patients reflected on-going recruitment as genotyping were performed at

different time points. There is an overlap between the clinical cohorts in chapters 3 and 4, but these are addressed separately in the respective chapters. All patients were unrelated. A further replication case-control cohort from Sweden was also used and recruited from the Gastroenterology Units, Karolinske Institute, Sweden. All patients and controls were coded anonymously prior to entry to database as described below. In each of these patients, 10 mls of whole blood were obtained for DNA extraction (method described in following subsection).

4.1.4 Phenotypic data acquisition

Phenotypic data was extracted using standard proforma datasheets (see Appendix). In patients with acute severe UC, a further total of 56 parameters where collected for patients during the acute phase of illness in order to investigate their respective discriminant value in predicting outcome during an attack of severe UC (n=167). Further phenotypic classifications are based on the Vienna and Montreal disease classifications. In the latter, this classification has allowed the further subcategorisation of UC by disease extent (E1-proctitis, E2-left-sided and E3-extensive disease) and severity (S3-severe disease as defined by the modified Truelove and Witts criteria. The descriptions of the current cohort in the association studies will be presented in detail in Chapters 7 and 8.

5.1.5 Database/Statistical Programs

All original phenotypic data was stored in Access Database, which was independently maintained by a database manager (Hazel Drummond). Additonal data sorting was

performed using the Excel Database. The main statistical analyses were performed using the Minitab version 10, GraphPad Instat and Prism programs. Log-likelihood analyses were described in detail in the relevant chapters, and carried out using EH+ and partition-ligation expectation-maximization (PLEM) programs accessed via the Medical Reseach Council-Rosalind Franklin Centre of Genomic Research (www.rfcgr.mrc.ac.uk). Haplotype construction/analyses were performed using Haploview 3v2, Cocaphase Software and also SNP-HAP (latter 2 progrmas accessed at www.rfcgr.mrc.ac.uk). The re-sequencing scoring was undertaken using the Sequencher software (Gene Codes Corporation, Ann Arbour, Michigan, USA).

2.1.5 Genomic Databases

The sequence variations to design SNP primers were derived from the Ensembl (www.ensembl.org), HapMap (www.hapmap.org) and NCBI Genbank sequence databases (http://www.ncbi.nlm.nih.gov/Genbank/index.html). In addition, the Haploview program (http://www.broad.mit.edu/mpg/haploview/) was used to assess linkage disequilibrium.

2.1.6 Ethics Approval

The Lothian Research and Ethics Committee (LREC) approved this study and written consent was obtained from all patients.

2.2 Cellular methods: Genotyping

1.2.1 DNA extraction and storage

Genomic DNA was extracted from peripheral venous blood by a modified salting-out technique and resuspended in 1xTE (10mM Tris (pH 8.0), 1mM EDTA (pH 8.0) at a

final concentration of 100 ng/ml. The optical density of the resultant DNA was measured using a Gene Quant Pro instrument to estimate the actual DNA concentration. Using a standard protocol, 10 mls of whole blood were added into a 50ml conical tube together with 40 mls of red cell lysis buffer, and mixed and resuspended for 5 minutes. This was then centrifuged at 3000 rpm for 10 minutes. Following this, the supernatant was removed, the pellet resuspended with 10 mls of RCLB and re-centrifuged at 3000 rpm for a further 5 minutes. The pellet was then further resuspended in 3 mls of nuclear lysis buffer and sodium dodecyl sulphate. One ml of 6M sodium chloride and 3mls of chloroform were subsequently added leading to emulsification of DNA (after vigorous mixing). This was then centrifuged at 3000 rpm for 20 minutes. The contents of the tube separate into 3 layers for which the middle was that of DNA material. This layer was then removed and added into ethanol to allow precipitation of DNA. This DNA pellet was then removed and washed in 10mls of 70% ethanol and then dried in room air for 5 minutes. The precipitated DNA was transferred into 0.5 mls of TE buffer and stored at 4°C until dissolves. Genomic DNA is diluted to 100ng/μl for PCR analysis.

2.2.2 PCR reaction: TaqMan technology

The main genotyping in this thesis was performed using TaqMan® (ABI, San Diego, CA). This PCR-based assay uses laser scanning technology which excites fluorescent dyes present in the specially designed TaqMan® probes. The system includes a built-in thermal cycler, a laser to induce fluorescence, CCD (charge-coupled device) detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. The cycle-by-cycle detection of the increase in the amount of PCR product is quantified in real time as the special probes, "reporter dye",

fluoresces when the "quencher" is removed from the fragment during the PCR extension cycle. The primers for respective SNPs studied in this study are selected using the generic Assay-on-Demand or Assay-by-design service by ABI, Inc. The prepared genomic DNA was laid-out in 96-well plates prior to genotyping at the Wellcome Genetics Core, Western General Hospital, Edinburgh. In each plate, negative control wells are used to check the quality of reaction.

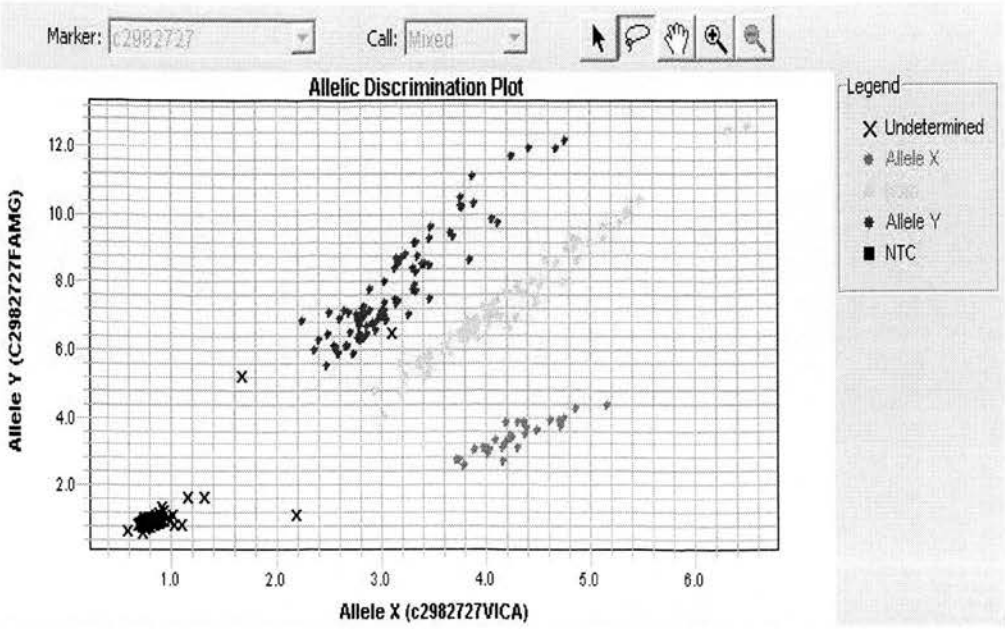
TaqMan reaction volumes and cycling conditions

Reaction Component	Assay by Design 40X mix
Abgene Absolute QPCR ROX mix	2.5µl
Genotyping Assay Mix	0.125µl
dH_2O	2.375µl

Thermal cycling conditions were two initial holds (50° C for 2 min and 95° C for 10 min) followed by a 40-cycle two-step program (95° C for 15 sec and 60° C for 1 min).

3.2.3 Allelic discrimination in TaqMan

Allelic discrimination was scored independently by 2 blinded-laboratory scientists (Angie Fawkes and Stuart Bayliss, Wellcome Genetics Core, Edinburgh). As quality control, a random selection of DNAs was re-genotyped and compared with original data. An example of the allelic discrimination plot was as shown below.



4.2.4 DNA sequencing reaction

PCR reaction mix (14 µl of PCR mix as described below and 2 µl DNA gives a final volume of 16 µl). The components were assembled in 1.5 ml eppendorf using filtered tips. The cycling conditions for the sequencing PCR reaction was 25 cycles of 96°C (10 seconds), 50°C (5 seconds) and 60°C (4 minutes).

	x 1	**x 100 (one plate)**
MQ water	9.952	995.2
Qiagen buffer	1.6	160
25 mM $MgCl_2$	0.64	64 (final concentration 2.5mM)
dNTP mix (25 mM each)	0.128	12.8
Qiagen HotStart Taq	0.08	8.0
Forward primer (10 µuM)	0.8	80
Reverse primer (10 µuM)	0.8	80
Total volume	14.0	1400

2.2.5 PCR clean-up and re-sequencing protocol

Following confirmation of PCR products, 4 µl of Exo-Sap was added to the remaining 10 µl of the PCR products. The reaction plate was then put into the PCR machine with this cycling parameter: 37°C (45 minutes), 80°C (15 minutes) and 4°C (hold). The 80°C temperature will inactivate most of the enzyme. However, Exo-Sap will still be active in 4°C; therefore if the plates were not used immediately, these were stored at -20°C. Two µl of this cleaned-up PCR were used in direct sequencing as detailed below.

To clean up the sequencing reaction products, 80 µl of 80% isopropranol were added to each reaction, mixed thoroughly and left in room temperature for 10 minutes. Thereafter, the PCR plates were centrifuged at 3780 rpm for 60 minutes. Following this process, the PCR plates were inverted on a blotting paper and centrifuged for 1 minute at 1000 rpm. The whole step was repeated once more; this time 150 µl of 70% isopropranol was added to each reaction and centrifuged at 3870 rpm for 10 minutes. Following further inversion of the plates and low speed centrifugation, the plates were left to dry in room temperature for 15 minutes.

2.2.6 Equipments and reagents

PCR machines

TaqMan ABI Fast Real Time PCR 7600 HT (Applied Biosystems)

ABI 3700 Big Dye Terminator Sequencer (Applied Biosystems)

Techne Touchgene PCR Gradient machine

Electrophoresis reagents

Agarose gel:

Multipurpose agarose (2.25g) was added to 150 millilitres (ml) of half strength TBE into a 250 ml beaker. This was then microwaved on full power for between 2 and 3 minutes agitating half way through until all of the agarose had dissolved into solution. The agarose was then cooled to around 50°C by running under a cold water tap, before 10 microlitres (µl) of ethidium bromide was added and then agitated further to ensure even distribution. The agarose solution was then poured into a transparent gel-casting tray, which was sealed at each end using autoclave tape. Four combs containing either 12 or 16 wells were then inserted into the gel. The gel was then left to cool and solidify at room temperature for at least 30 minutes.

Loading Buffer:

Orange G in 40% sucrose was used aas loading buffer. 5µl was added to each PCR well prior to loading.

Agarose gel electrophoresis

100mls of liquid agarose was poured into a transparent gel-casting tray, which was sealed at each end using autoclave tape. Twelve combs, each comprising 12 wells, were placed into their slots and the gel left to solidify for 30 minutes at room

temperature. The tape and combs were removed before placing in a horizontal electrophoresis tank filled with 0.5x TBS. Prior to pipetting, PCR reaction was pre-mixed with Orange G loading buffer. 10µl of PCR mix was then loaded from each well into the gel. The samples were electrophoresed for 30 minutes (150 V, 150 mA, 150 W).

Interpretation of gel images

Gels were photographed digitally under UV light (320nm) using a 3 second exposure. Images were anonymised and stored on a computer network for later genotyping. All digital images were read manually. Each PCR reaction was deemed to have worked if the control amplicon was present. Positive allele-specific amplification was identified by the presence of a correct sized PCR amplicon. If a reaction had neither control nor allele-specific amplicon the reaction was deemed 'not tested'. For single markers two reactions (one detecting the wild type and the other the polymorphism) were read simultaneously.

2.2.7 Primer/Probe selection

ABCB1/MDR1 gene

The G2677T/A (rs2032582) was a triallelic SNP, and allelic/genotypic data was obtained by seqeuencing using GTCTCATGAAGGTGAGTTTTCAG (forward primer) and GGGAGGAAGGAAGAACAGTGT (reverse primer) to flank a 572-base pair region (product size 572 base pairs) (Chapter 5).

Two further SNPs, rs1186746 rs1186745 (located 13 base pairs from each other, intron 27) could not be genotyped using TaqMan due to technical reasons and these SNPs were sequenced using the TAGTTCTTTTCCCTAATTCCTCTT (forward primer) and GGTTCACACCATTCTCCTG (reverse primer) (product size 246 base pairs) (Chapter 6).

The remainder tSNPs for ABCB1/MDR1: rs3789243 (Intron 3), rs1128503 (Exon 12_1), rs2235046 (Intron 16) and rs1045642 (Exon 26) were available as Assay-on-Demand service by ABI-biosystems.

Pregnane-X receptor (PXR)

A full set of five tSNPs (rs1523127, rs2461823, rs7643645, rs1464603 and rs2472682) were available on Assay-on-Demand service by ABI-biosystems.

Multidrug resistant protein 3 (MRP3)

Eight tSNPs (rs757421, rs2412333, rs739921, rs12051822, rs2301837, rs879459, rs2277624 and rs3785911) were on Assay-on-Demand service by ABI-biosystems. All primers were checked with BLAST program (www.ensembl.org).

Chapter 3

The efficacy of corticosteroid therapy in inflammatory bowel disease: analysis a 5-year UK inception cohort

Abstract

Background: Corticosteroids remain the mainstay of first-line therapy in active inflammatory bowel disease.

Aims: To determine the clinical outcome after the first corticosteroid-therapy and; to identify factors which predict response/failure.

Methods: 216 (136 UC and 80 Crohn's disease) patients were identified in this 5-year inception cohort. The outcomes of early (30-days) and late (1-year) response were used. Multivariate analyses were performed to identify factors associated with outcome.

Results: 86 (63%) and 60 (75%) UC and Crohn's disease required corticosteroid therapy respectively. In UC, at 30-days, 69 (51%), 42 (31%) and 25 (18%) patients demonstrated complete response, partial response and no response respectively. For Crohn's disease, these outcomes were observed in 32 (40%), 28 (35%) and 20 (25%). After 1 year, 75 (55%), 23 (17%), and 29 (21%) patients with UC demonstrated prolonged response, corticosteroid-dependence or required surgery respectively. For Crohn's disease, these outcomes were observed in 30 (38%), 19 (24%) and 27(35%) patients respectively. Extensive UC was a predictor of surgery (p=0.001, OR 15.24). In Crohn's disease, inflammatory disease behaviour was negatively associated with surgery (p=0.02, OR 0.13).

Conclusion: Although corticosteroids are effective, dependence/resistance remains common. Patients with extensive UC and fistulising/stricturing Crohn's are most at risk of failing corticosteroid-therapy.

3.1 Introduction

For more than 50 years, corticosteroids have represented the mainstay of medical therapy in active inflammatory bowel disease (IBD)(Truelove SC and Witts LJ 1955). As with many inflammatory conditions(Barnes 2004; Chikanza and Kozaci 2004; Seki M et al. 1998; van Schaardenburg D et al. 1995; Carmichael et al. 1981), failure to respond (corticosteroid resistance) and to wean (corticosteroid dependence) are significant problems; contributing to increasing morbidity secondary to both continually active disease and prolonged exposure to corticosteroid therapy(Farrell and Kelleher 2003; Laine and Hanauer 2003). The increased awareness of the detrimental effects of prolonged corticosteroid use has led to stringent attempts by patients and physicians alike to minimise the exposure or indeed to avoid the use corticosteroids all together. In this context, the use of immunosuppressants such as azathioprine/6-mercaptopurine and methotrexate are now also firmly established(Pearson et al. 2000; Sandborn et al. 2000; Jewell and Truelove 1974; Fraser, Orchard, and Jewell 2002; Feagan et al. 1995; Feagan et al. 2000; Ardizzone et al. 2006). In the recent years, there has been an expansion of biological therapies, in particular, monoclonal antibody therapy directed against tumour necrosis factor such as infliximab in Crohn's disease(Sands et al. 2004; Hanauer et al. 2002) and more recently in UC(Rutgeerts et al. 2005; Jarnerot et al. 2005b); together with nutritional therapies(Gonzalez-Huix et al. 1993; Heuschkel 2000; Zachos, Tondeur, and Griffiths 2001).

As described in chapter 1, several studies have attempted to define the prevalence of corticosteroid resistance in IBD. By defining corticosteroid dependency as initial response to an adequate dose of corticosteroid but followed by clinical relapse at dose

reduction or within 30 days after corticosteroid treatment and steroid resistance as no response within 30 days of corticosteroid therapy, studies have shown rates of dependency and resistance of 30% amongst patients with IBD(Munkholm et al. 1994; Faubion et al. 2001). A high frequency of surgical intervention was reported in corticosteroid dependent (26%) and resistant patients (59%) within 1 month after corticosteroid treatment(Munkholm et al. 1994). Although factors such as underdosing and abrupt tapering of corticosteroids maybe responsible for failure of this therapy, a prospective study involving 48 consecutive patients with active CD, using a higher oral corticosteroids dose and a slower tapering schedule than used in the previous studies reported steroid dependency and resistance rates of 63% and 13%, respectively(Reinisch et al. 1995). I report the rates of corticosteroid resistance and dependence in a more recent 5-year hospital-based inception cohort. In order to provide an accurate comparison, we have utilised previously used definitions in the studies by Munkholm and Faubion et al. I have assessed the 30-day and 1-year outcome of the first corticosteroid treatment for active Crohn's disease and UC in this cohort. This is the first UK dataset in inflammatory bowel disease and the only the second study worldwide in UC describing the natural history of corticosteroid therapy. In addition to the descriptive results, I provide detailed analyses of clinical parameters which may identify patients at increased likelihood of early non-response within 30 days of therapy and surgery within 1-year of follow-up.

3.2 Material and Methods

The data in this study was based on a 5 year hospital-based inception cohort study of IBD patients diagnosed at the Western General Hospital, Edinburgh, Scotland (1998-2003). All patients were either referred by their General Practitioners or presented as

emergencies. This hospital serves an estimated catchment area of 448, 624 persons in the Lothian region, Scotland, United Kingdom. Tertiary referrals outwith the Lothian region hospitals were not considered.

Patient identification

I identified patients who were newly diagnosed between 01/01/98-15/08/2003 using 2 methods: Firstly, we analysed the Inflammatory Bowel Disease Database, Western General Hospital, Scotland. This is a continually updated database which is managed independently by a dedicated database manager (HD) and represents the core resource of the IBD genetics programme in the Western General Hospital. Secondly, we searched the Histopathological Database of the Lothian University Hospitals (incorporating Western General Hospital) (SNOMED) using the term INFLAMMATORY BOWEL DISEASE. All case notes were reviewed and, a total of 136 cases of UC and 80 cases of CD which fitted the above criteria were identified. Information was extracted by reviewing relevant outpatient letters, referral letters and discharge summaries in addition to biochemistry, radiology, colonoscopy and histology reports. Patients with the diagnosis of indeterminate colitis were excluded from the study.

Data collection

All relevant data were collected and entered into a standard proforma data sheet. Among the details collected were age at diagnosis, sex, date of commencement of corticosteroid treatment, smoking details, concomitant medication within 30 days of diagnosis, site and extent of disease. UC phenotype was classified by disease extent, severity and need for surgery. CD was classified according to the Vienna Classification of disease location, behaviour and age of diagnosis, as previously described (Gasche et al. 2000).

Case definitions

Corticosteroid therapy

Corticosteroid therapy consisted of either oral/intravenous Prednisolone or oral Budenoside. Date of corticosteroid commencement, interval between diagnosis and corticosteroid treatment, date of discontinuation, length of treatment and reason for discontinuation were documented when the first corticosteroid therapy was started (all patients in this study are corticosteroid naïve). Subsequently, outcomes at 30 days and 1 year after commencement of corticosteroid therapy were assessed.

Dose and taper of corticosteroids

Corticosteroid therapy consisted of either intravenous/oral Prednisolone or oral Budesonide. In patients requiring intravenous treatment, the intended duration of corticosteroid therapy was 5-7 days with conversion to oral Prednisolone at 40 mg/day if clinical response was evident. Our centre uses intravenous methylprednisolone 60 mg/day (equivalent dose of 84 mg of oral Prednisolone). The policy for tapering doses of oral corticosteroids was to decrease the dose of Prednisolone by 5 mg/week following a usual starting dose of 40 mg/day of one week. In patients with Crohn's disease requiring oral Budesonide, the starting dose was 9 mg/day and tapering by 3 mg every 3 weeks was performed.

Outcomes/Endpoints

Outcomes and end-points of follow-up were considered at day 30 and 1 year.

At 30 days: Patients were classified into complete remission, partial remission and no response.

1. Complete Remission:

Total regression of clinical symptoms, (≤2 bowel movements per day, no blood, pus or mucus in stool and no abdominal pain, fever, weight loss or extra-intestinal symptoms)

2. Partial Remission:

Regression of clinical symptoms, (≤4 bowel movements per day, blood, pus or mucus in stool or abdominal pain or all 4 less than daily and no systemic symptoms such as fever, weight loss or extra-intestinal symptoms)

3. No Response:

No regression of clinical symptoms.

At 1 year: After the initial dose of corticosteroid, patients were again classified into 1 of 3 categories: Prolonged response, corticosteroid dependent and Surgery

1. Prolonged corticosteroid response:

Maintenance of complete or partial remission for at least 30 days after treatment has completed

2. Corticosteroid dependence:

Initial response to an adequate dose of corticosteroid but followed by clinical relapse at dose reduction or within 30 days after corticosteroid treatment

3. Surgery:

No response

Statistical Analysis

Descriptive statistics were displayed using median values and interquartile range. Univariate analyses were performed to compare variables between responders/non-responders within 30 days of corticosteroid therapy and surgery/non-surgery 1 year after initiation of corticosteroid therapy. Categorical and continuous variables were

analyzed using Chi-square test and Mann-Whitney tests respectively. Odd ratios (OR) were given with 95% confidence intervals and 2-sided p-values. A p-value of ≤ 0.05 was considered significant. Kaplan-Meier survival curve analyses and log-rank test were performed to demonstrate and compare the cumulative risk of surgery in UC and Crohn's Disease respectively, using GraphPrism (version 4.00 for Windows, GraphPad Software, San Diego California USA). Multivariate analysis was performed using a logistic regression model to test identify clinical parameters which predict the non-response to corticosteroid therapy within 30 days; and the need for surgery within one-year of the initiation of corticosteroid therapy respectively. The logistic regression analyses were performed in the groups of UC and Crohn's Disease separately. For UC, the variables considered were: sex, age at diagnosis, smoking, disease extent, the use of 5-ASA/mesalazine and azathioprine. For Crohn's Disease, the similar variables were used with disease location and behaviour replacing disease extent. All the above analyses were performed using the Minitab statistical software (version 13.20, USA).

3.4 Results

Patient demographics (Table and Figure 1)

86 (63%) UC and 60 (75%) CD patients required treatment with corticosteroids (Figure 1). The demographics and phenotypic details are summarized in Table 1. In UC, 64 (74%) and 22 (26%) patients were started on oral and intravenous corticosteroids respectively (Figure 1). Sixty (75%) patients with CD were initially treated with corticosteroids. Of these, 52 (87%) and 8 (13%) patients were started on oral and intravenous corticosteroids respectively (Figure 1). Only one patient was treated with intravenous ciclosporine in this cohort.

Immediate outcome (30 days) (Figure 2)

UC

Of the 86 UC patients, 44 (51%) achieved complete remission, 27 (31%) were in partial remission and no response was seen in 15 patients (18%). Of the 15 who did not respond, 6 (40%) proceeded to have surgery within the 30 days, after a median of 8.5 days (IQR: 5.8-14.8 days).

CD

Of the 60 CD patients, 24 (40%) achieved complete remission, 21 (35%) were in partial remission and no response was seen in 15 (25%). Of the 15 who did not respond, 4 were hospitalised, 3 of them were commenced on intravenous corticosteroids and 1 patient, on infliximab. In the non-responder group, 2 subsequently required surgical intervention within the 30 days, after 5 and 28 days respectively.

Effect of corticosteroid dose/taper on immediate outcome

There were no statistical significant differences in the starting dose of corticosteroids (Prednisolone, mg ±IQR) between the groups of complete response (40 mg; 30.5-40.0), partial response (40mg; 20-40) and need for surgery (40 mg; 27.5-40.0) respectively; in UC (p=0.30). The similar trend was evident in CD: complete (40 mg; 20.0-40.0), partial (40mg; 30.0-40.0) and surgery (40 mg; 32.5-40.0) respectively (p=0.78). All patients requiring intravenous corticosteroids received similar dosing schedule (60 mg/day of methylprednisolone). The retrospective nature of this dataset precluded the ability to determine whether different tapering schedules determined early or late outcomes.

One year outcome (Figure 2)

In UC, 47 patients (55%) were found to be in prolonged response without corticosteroid therapy, 15 (17%) and 18 (21%) patients were corticosteroid dependent and required surgery respectively. In CD, prolonged response was seen in 23 patients (39%). 14 patients (23%) and 21 (35%) patients were corticosteroid-dependent and required surgery respectively.

Surgery at one year (Table 2)

UC

Overall, eighteen patients (21%) required surgical intervention. Fifteen patients had undergone proctocolectomy with ileostomy and the remaining 3 had proctocolectomy with ileal pouch formation. The indications for surgery were shown in Table 2. In total, 9 patients failed to respond to intravenous corticosteroids during an acute flare of severe UC (1 patient demonstrating clinical signs of toxic dilatation). The median

duration of corticosteroid therapy prior to colectomy was 9 days (IQR 7-15 days). Nine further patients required surgery in a semi-elective fashion due to chronic continuous symptoms. Overall, no patients experienced any major operative complications (e.g. pelvic sepsis or anastamotic leak requiring further laparotomy).The median time between CS commencement and surgery is 0.25 years (IQR: 0.03-0.56 years).

CD

Twenty-one patients (35.0%) needed surgery. Seventeen (28.3%) patients had a resection of the ileocaecal segment with anastomosis, 2 (3.3%) patients required colectomy with permanent ileostomy and a further 2 (3.3%) required surgical drainage of perianal abscesses. In this cohort, the median number of days between initiation of corticosteroid therapy and surgery is 0.39 years (IQR: 0.21-0.68 years). Survival curve estimates of surgery within 1-year of corticosteroid therapy show that the rate of surgery tend to be higher in patients with CD compared with UC, p=0.08, hazard ratio = 2.05, 95% CI 0.94-3.85 (log rank test) (Figure 3).

Univariate analysis: Factors influencing response to corticosteroids

In UC, there were no significant differences in the clinical parameters examined between the responders and non-responders within 30 days (Table 3a). Following one year of corticosteroid therapy initiation, 11 (55.0%) patients with extensive disease required surgery compared with 7 patients with left sided disease (10.8%) (p=0.0001, odds ratio 10.13, 95% CI 3.11-32.95). In CD, there were no significant differences in the clinical parameters examined between the responders and non-responders within 30 days (Table 3b). Following one year of corticosteroid therapy initiation, two significant differences were observed. Firstly, chi-square test for independence

demonstrated a significant difference in the need for surgery according to the 3 disease locations (colonic 58.3% vs. 41.7%, ileal 62.5% vs. 37.5% and ileo-colonic 21.9% vs. 79.1%) (p=0.0009). Secondly, the use of 5-aminosalicylate compound therapy appeared highly protective against surgery (p=0.005, odds ratio 0.10, 95% CI 0.02-0.51).

Multivariate analysis

I have derived 2 separate models for the respective conditions of UC and CD, using the outcome of non-response within 30 days; and surgery within one year of the initiation of corticosteroid therapy as the dependent variable (Table 4). The similar variables examined in the univariate analyses were used in these binary logistic regression models.

In UC, extensive disease at diagnosis for UC (extent > splenic flexure) was predictive of surgery within one year of corticosteroid therapy initiation with a highly significant association (p=0.001, odds ratio 15.24, 95% CI 3.72-62.37). No other clinical parameters in both models demonstrated significance. In CD, a significant negative association between 'inflammatory' disease behaviour phenotype and surgery (p=0.02, odds ratio 0.13, 95% CI 0.02-0.70). The highly significant negative association between 5-ASA therapy and surgery observed on the univariate level was not significant when adjusted for other variables (p=0.09).

3.4 Discussion

This data first and foremostly, emphasise the importance of corticosteroid resistance/dependence in the management of patients with Crohn's disease and UC. These observations thereby highlight the real clinical need to understand the molecular mechanisms behind this phenomenon. Equally, it is also clear that corticosteroid therapy can remain efficacious and safe in a proportion of patients. Thus, a subset of patients will inherently respond poorly to corticosteroids: in our analyses, the subphenotypes of disease such as extensive UC and stricturing/fistulising Crohn's disease at presentation appear to be associated with this phenomenon.

It is of interest to observe that the prevalence of corticosteroid resistance and dependence appear stable across time and population. This is clearly evident when data from our inception cohort (patients diagnosed between 1998 and 2005) are compared with the population-based based datasets from Stockholm and the Mayo Clinic (1979-1983 and 1970-1993 respectively) (Table 5). The overall use of corticosteroids was higher in our study, 63% in UC and 75% in CD; when contrasted with 34% in UC and 43% in CD, and 56% in CD in studies by Faubion (Faubion et al. 2001) and Munkholm(Munkholm et al. 1994) respectively. This reflects the hospital-based nature, and conceivably a more 'severe' spectrum of presentation in this cohort. As with other inflammatory diseases(Barnes 2004; Barnes 1998; Seki M et al. 1998; van Schaardenburg D et al. 1995), it would appear that corticosteroid resistance and dependence occur irrespective of disease and subphenotype – thus the similar results in our study as compared with the older population-based studies. This argument is

even more persuasive given the similar trends observed in the paediatric UC population (Hyams et al. 2005).

With the present study, the overall rates of azathioprine usage were higher than those reported from the cohorts from Mayo Clinic, (28% vs. 0% in UC and 8% vs. 3% in CD) (Table 4). However, even the observed rates in our study may be an underestimate of the current practice in 2006, as our data from 1998-2003 were reflective of the transitional period in UK before the increasingly widespread use of *early* immunosuppressive therapy, a very recent change in clinical practice. Despite the increased use of azathioprine, there were no overall differences were seen in the rates of surgery. The use of 5-aminosalicylate compounds was similar in this study (72% vs. 77% for UC; and 49% vs. 50% in CD in our and Faubion's cohorts respectively).

Our multivariate analyses suggest that extensive UC is the only significant predictor of surgery within 1-year of corticosteroid therapy (p=0.001, odds ratio 15.24, 95% CI 3.72-62.37). This observation is consistent with the findings of Jarnerot et al., whose data suggested that extent and severity of disease correlate with need for surgery(Jarnerot, Rolny, and Sandberg-Gertzen 1985). In Crohn's disease, inflammatory disease behaviour phenotype demonstrated a significant negative association with surgery. The protective effect of 5-aminosalicylate compounds therapy as shown in Faubion's study was once again demonstated in the current study(Faubion et al. 2001).

Recently, much enthusiasm has been directed towards approaches in 'positioning' new and perhaps more efficacious and better tolerated therapies in the traditional pyramid of medical therapy in IBD. With the established use of biological therapies such as infliximab in Crohn's disease, there is also now suggestion of 'inverting' this pyramid – by giving the most efficacious therapy early in the course of active disease thus allowing patients the maximum chance of early response/remission and minimizing exposure to corticosteroid therapy(Hanauer 2005). Given the consistent problems of corticosteroid resistance and dependence(Faubion et al. 2001; Munkholm et al. 1994; Jarnerot, Rolny, and Sandberg-Gertzen 1985; Hyams et al. 2005); do the current data now therefore lend its full-hearted support to this approach?

It is noteworthy that in UC and Crohn's disease, the subsets of patients with extensive UC or fistulizing/stricturing disease behaviour respectively, are at increased likelihood to require surgery within 1-year of corticosteroid therapy following diagnosis. Intuitively one might suggest that it is these patients who should be considered as candidates for an early aggressive approach with immunomodulatory therapy as the likelihood of corticosteroid therapy failure is increased. However, the clinical decision-making in the adoption of 2nd line medical or immunosuppressive therapy remains complex, requiring robust evidence from good clinical trials.

In this context, Hommes et al. recently presented data comparing 'top-down' (initial therapy with infliximab and azathioprine) and 'step-up' (initial therapy with corticosteroids) in Crohn's disease (Hommes DW et al. 2005). In this study, no differences were seen in the rates of clinical remission between these 2 groups (77% and 76% respectively at 1-year follow-up) but the proportion of patients free from

corticosteroids use at 1-year were higher in the former group (76% vs. 64%). The finding of mucosal healing was also higher in the 'top-down' group (75% vs. 21%) but there were no differences in the rate of adverse events. Therefore, the short term clinical impact of a front-line aggressive strategy in Crohn's disease at least may not be as significant as initially anticipated. In UC, on the other hand, Ardizzone et al. had recently clearly demonstrated a benefit in the use of Azathioprine in corticosteroid dependent patients with UC as compared with the use of 5-aminosalicylate compounds (Ardizzone et al. 2006). Patients treated with azathioprine demonstrated significantly higher rates of clinical and endoscopic remission (odds ratio 4.78, 95% CI 1.57-14.5).

It is of course important to consider whether lack of response of corticosteroids may be attributed to the size of the initial dose used and the speed of corticosteroid withdrawal rather than a subset of inherently resistant or dependent patients. While poor response to corticosteroids may be influenced by such factors as underdosing and abrupt tapering, a prospective study in 48 consecutive patients with active Crohn's disease using a higher oral corticosteroid dose and a slower tapering schedule than used in the previous studies reported steroid dependency and resistance rates of 63% and 13% respectively(Reinisch et al. 1995). In acute severe UC patient group (167 patients, data presented in the following chapter), our recent Edinburgh data show that despite high intravenous corticosteroid doses, 40% will still fail to respond and require colectomy (Ho et al. 2004). In this present study, there were no significant differences in the starting doses of corticosteroid between each of the outcome groups within 30-days of initiation. Drawing parallel critical analyses with many other inflammatory conditions, our data suggest that inherent resistance to corticosteroids

represents the more significant problem than dosing/tapering(Barnes 2004; Barnes 1998; Seki M et al. 1998; van Schaardenburg D et al. 1995).

There are some limitations to this study . Firstly, the time-points chosen at 30-days and 1-year in the context of a retrospective analysis, may introduce a degree of bias. Secondly, the time span between these 2 points may be too wide to accurately record the corticosteroid requirements and response in between. Thirdly, the clincial decision to introduce second-line medical therapy such as Azathioprine which may influence outcomes at 1-year may not be universally applied.

However, it remains pertinent that a large proportion of patients in IBD will respond to corticosteroids well. Therefore, the important current challenges include the need to identify patients with likely to respond poorly to corticosteroids and to understand the molecular mechanisms behind the apparent 'refractoriness' to corticosteroids. In addition to the clinical parameters implicated in this study, progress in genomic and proteomic are awaited. Compelling studies of P-glycoprotein 170 expression(Farrell et al. 2000), increased expression in the beta-splice variant of the glucocorticoid receptor(Honda M et al. 2000) and T-cell subsets corticosteroid resistance(Hearing et al. 1999) have not yet been fully translated into clinical practice. In terms of pharmacogenetics, most pertinently the multidrug resistance gene 1 (MDR1) polymorphisms and the rare HLA DRB1*0103 allele appear to predict severe disease(Ho et al. 2005; Potocnik et al. 2004; Bouma et al. 1999; Roussomoustakaki et al. 1997; Duerr and Chesny IJ 1997).

In conclusion, this study demonstrates that corticosteroid resistance and dependence remains a significant problem in a proportion of patients with IBD. Patients with extensive UC or fistulizing/stricturing CD at diagnosis are most at risk of corticosteroid failure, and these may be optimum candidates for early aggressive medical approach. Notwithstanding this, corticosteroids can be a highly efficacious and cost-effective therapy in a sizeable proportion of patients. This study once again highlights the need to understand fully the mechanisms of corticosteroid resistance as the ability to reliably identify patients who will not respond to this therapy will undoubtedly lead to a new shift in pharmacotherapy in IBD and other chronic inflammatory diseases, allowing physicians to consider other therapies early and thereby reducing corticosteroid-related toxicity (Rhen and Cidlowski 2005).

Figure 1: The description of the 5-year inception cohort in Western General Hospital, Edinburgh between 1998 and 2005 and the rates of both oral and intravenous corticosteroid use.

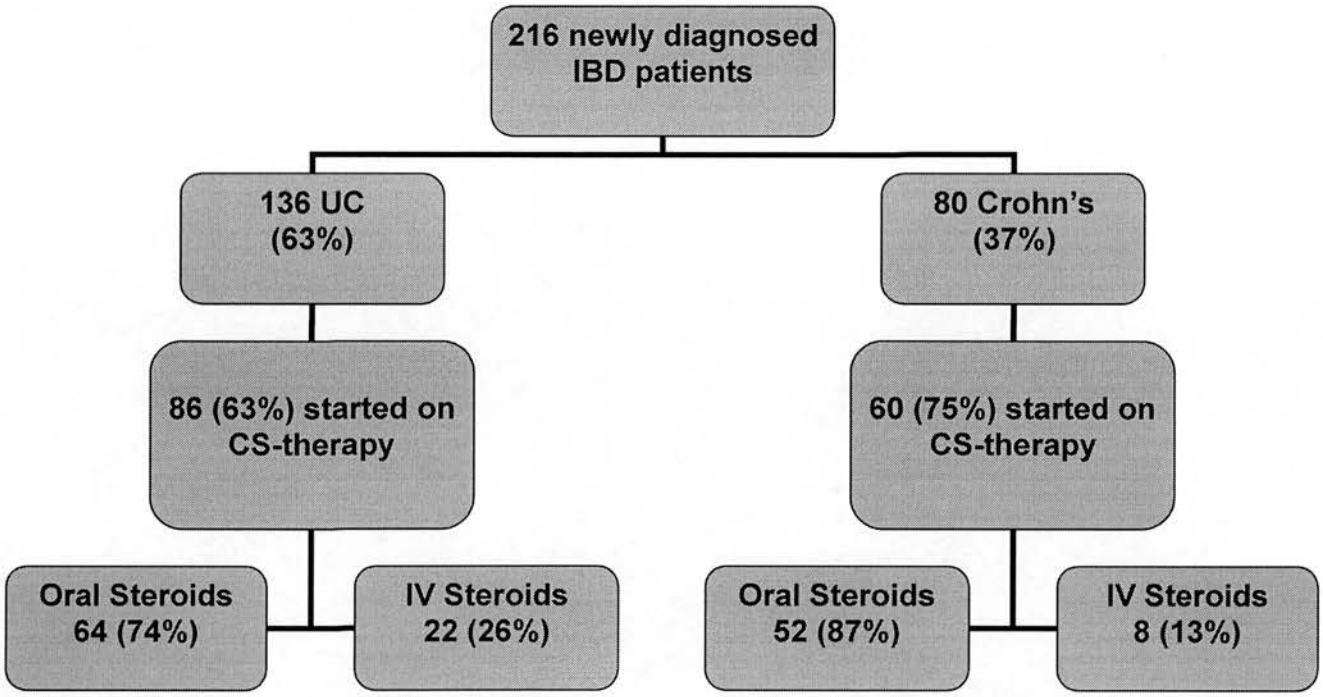


Figure 2: Outcomes at 30 days and 1 year for CD and UC

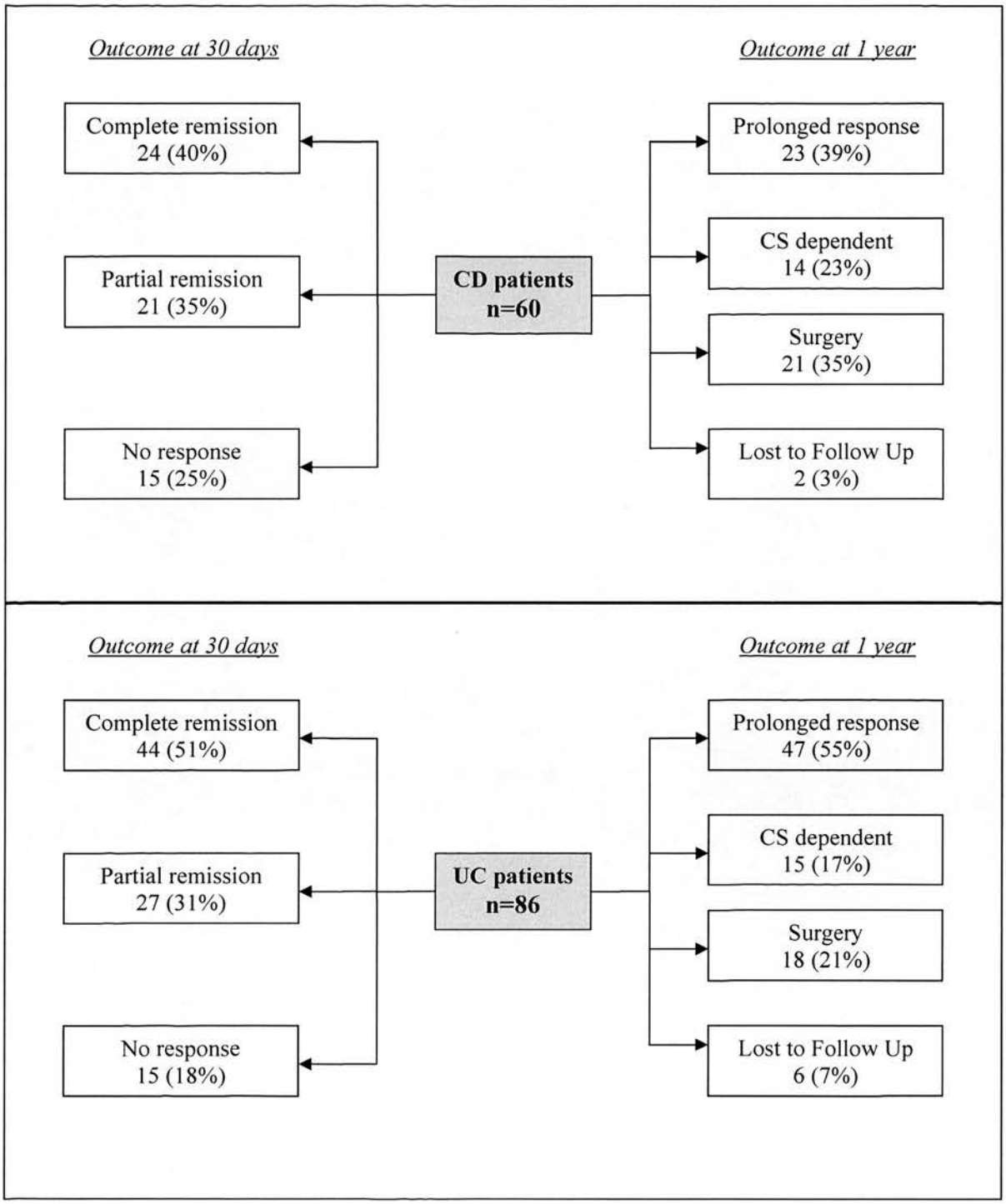


Figure 3: Cumulative risk of surgery over 1 year after starting CS therapy. In newly diagnosed patients with IBD and treated with corticosteroids, the rate of surgery is higher in patients with CD compared with UC; p=0.08, Hazard ratio = 2.05, 95% CI 0.94-3.85.

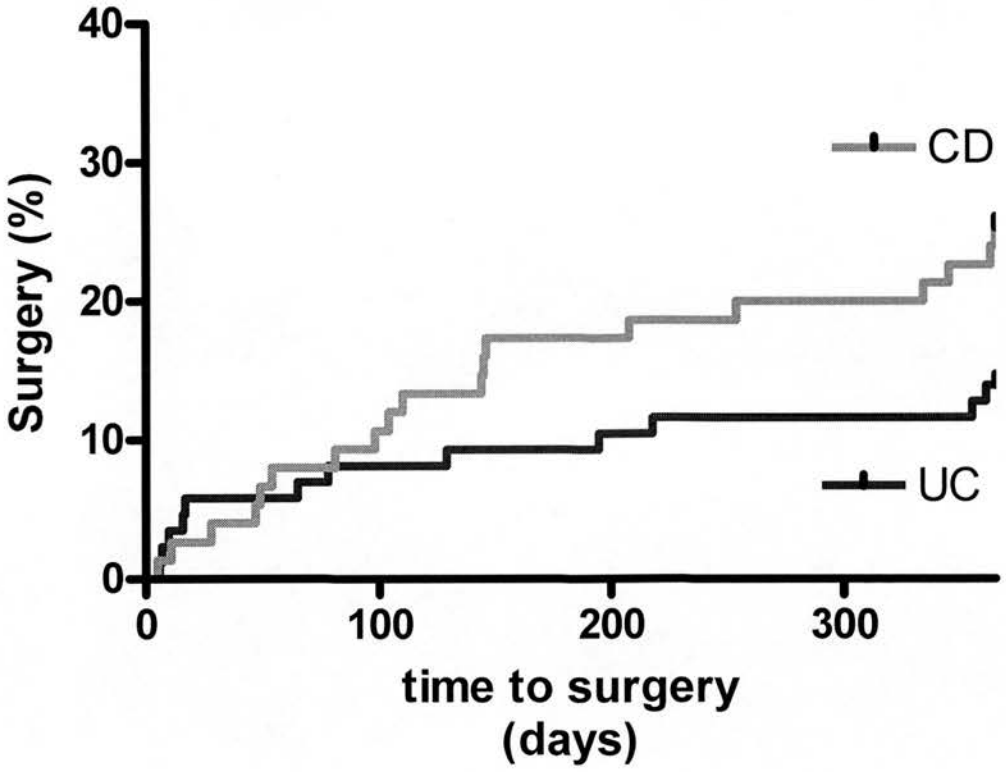


Table 1: Overall demographics of 86 UC and 60 CD patients commenced on corticosteroids on initial diagnosis. †Smoking status established at time of diagnosis. ‡Disease extent established at time of latest follow-up period (1 year). Extensive disease - >splenic flexure and Left-sided disease- < splenic flexure. □□ Disease location and behaviour determined at time of latest follow-up (1 year after diagnosis) according to the Vienna Classification(Gasche et al. 2000). CS- corticosteroids.

	UC (n=86)	**CD (n=60)**
M:F	44/42	22/38
Age at diagnosis (yr)	35.30 (IQR 24.40-50.95)	32.00 (IQR 25.10-57.25)
Duration on CS therapy (years)	0.23 (IQR 0.15-0.33)	0.31 (IQR 0.20-0.44)
†Smoking status Smokers Non-smokers	 9 (10.4%) 77 (89.6%)	 23 (38.3%) 37(61.7%)
‡Disease extent Left sided Extensive	 66 (76.7%) 20 (23.3%)	
□□ Disease location Ileal (L1) Colonic (L2) Ileocolonic (L3)	 -	 16 (26.7%) 12 (20.0%) 32 (52.3%)
Disease behaviour Inflammatory (B1) Stricturing (B2) Penetrating (B3)	 -	 42 (70.0%) 14 (23.3%) 4 (6.7%)
Mesalazine therapy (within 30 days of CS-therapy)	72 (83.7%)	49 (81.7%)
Azathioprine therapy (within 30 days of CS-therapy)	28 (32.6%)	8 (13.3%)

Table 2: Indications for surgery for UC and Crohn's disease.

UC	**N=18**
Failure to respond to corticosteroids in acute severe disease (corticosteroid resistance)	8
Toxic dilatation	1
Perforation/Major haemorrhage	0
Chronic continuous symptoms (corticosteroid dependency)	9
Crohn's disease	**N=21**
Failure to respond to medical therapy Ileal disease	17
Failure to respond to medical therapy Colonic disease	2
Failure to respond to medical therapy Perianal disease	2

Table 3a: Univariate analyses of clinical parameters between responders and non-responders (within 30 days); and surgery and no surgery (within 1 year of follow-up) for UC.

	Within 30 days		P-value OR 95% CI	Within 1 year		P-value OR 95% CI
	No response	Response (Partial and complete)		Surgery	No surgery	
Sex Females	22 (51.2%)	21 (48.8%)	1.00 1.10 0.47-2.56	8 (19.0%)	34 (81%)	0.79 0.80 0.28-2.27
Age at diagnosis (median + IQR years)	36.40 (25.90-54.90)	34.90 (24.30-46.60)	0.26	35.55 (24.95-51.18)	34.85 (23.20-56.05)	
†Active smoker Non-smoker	3 (33.3%) 40 (51.9%)	6 (66.6%) 37 (48.1%)	0.48 0.46 0.50-9.28	1 (11.1%) 17 (22.1%)	8 (88.9%) 60 (77.9%)	0.68 0.44 0.05-3.78
‡Disease extent Extensive disease Left sided disease	5 (25.0%) 9 (13.6%)	15 (75.0%) 57 (86.4%)	0.30 2.11 0.61-7.24	11 (55.0%) 7 (10.8%)	9 (45.0%) 58 (89.2%)	0.0001* 10.13 3.11-32.95
5-ASA therapy	34 (47.2%)	38 (52.8%)	1.00 0.96 0.39-2.39	13 (18.1%)	59 (81.9%)	0.16 0.40 0.11-1.38
Azathioprine therapy	15 (53.6%)	13 (46.4%)	1.00 1.04 0.42-2.57	4 (14.3%)	24 (85.7%)	0.40 0.51 0.15-1.73

Table 3b: Univariate analyses of clinical parameters between responders and non-responders (within 30 days); and surgery and no surgery (within 1 year of follow-up) for CD. [a] Chi-square test for independence in a 3 x 2 contingency table.

	Within 30 days		P-value OR 95% CI	Within 1 year		P-value OR 95% CI
	No response	Response (Partial and complete)		Surgery	No surgery	
Sex Females	21 (55.3%)	17 (44.7%)	0.59 0.71 0.24-2.08	12 (31.6%)	10 (69.4%)	0.10 2.60 0.88-7.68
Age at diagnosis (median + IQR years)	32.00 (25.10-58.00)	32.00 (24.00-57.00)	0.86	26.00 (20.25-51.75)	36.00 (28.00-58.00)	0.08
†Active smoker Non-smoker	7 (30.4%) 8 (21.6%)	16 (69.6%) 29 (78.4%)	0.54 1.59 0.48-5.18	9 (39.1%) 15 (40.5%)	14 (60.9%) 22 (59.5%)	1.00 0.94 0.32-2.73
‡Disease location Colonic Ileal Ileo-colonic	8 (66.6%) 11 (68.7%) 16 (50.0%)	4 (33.3%) 5 (31.3%) 16 (50.0%)	0.37[a]	7 (58.3%) 10 (62.5%) 7 (21.9%)	5 (41.7%) 6 (37.5%) 25 (79.1%)	0.009[a]
‡Disease behaviour Inflammatory Penetrating Stricturing	24 (57.1%) 3 (75.0%) 8 (57.1%)	18 (42.9%) 1 (25.0%) 6 (42.9%)	0.78[a]	13 (30.9%) 3 (75.0%) 8 (57.1%)	29 (69.1%) 1 (25.0%) 6 (42.9%)	0.07[a]
5-ASA therapy	28 (57.1%)	21 (42.9%)	0.75 0.76 0.20-2.95	15 (30.6%)	34 (69.4%)	0.005 0.10 0.02-0.51
Azathioprine therapy	2 (25.0%)	6 (75.0%)	0.06 0.20 0.03-1.05	1 (12.5%)	7 (87.5%)	0.13 0.18 0.02-1.57

Table 4a: Multiple logistic regression analyses of each potential clinical parameter for 30-day outcome of non-response for UC and CD.

UC (30 days outcome: non-response)	Coefficient	SE Coefficient	P-value	Odds ratio	95% CI	
					Lower	Upper
Sex Females	0.17	0.65	0.32	1.19	0.34	4.21
Age at diagnosis	0.004	0.02	0.79	1.00	0.97	1.04
†Active smoker	-20.00	-	0.99	0.00	0.00	∞
Extensive disease	0.56	0.69	0.42	1.74	0.45	6.75
5-ASA therapy	-1.08	0.68	0.11	0.34	0.09	1.28
Azathioprine therapy	-0.20	0.69	0.77	0.82	0.21	3.20
CD (30 days outcome: non-response)						
Sex Females	-0.41	1.18	0.17	0.66	0.15	2.87
Age at diagnosis	-0.006	0.02	0.74	0.99	0.96	1.03
†Active smoker	0.44	0.65	0.49	1.56	0.44	5.54
Disease location Ileal vs. Ileocolonic and colonic	0.11	0.81	0.89	1.12	0.23	5.45
‡Disease behaviour Inflammatory vs.Penetrating/Stricturing	0.27	0.71	0.70	1.31	0.33	5.25
5-ASA therapy	0.64	0.99	0.52	1.89	0.27	13.36
Azathioprine therapy	-0.25	0.97	0.79	0.77	0.11	5.24

Table 4b: Multiple logistic regression analyses of each potential clinical parameter for 1-year outcome of surgery for UC and CD.

UC (1 year outcome: surgery)	Coefficient	SE Coefficient	P-value	Odds ratio	95% CI	
					Lower	Upper
Sex Females	0.01	1.2	0.98	1.01	0.28	3.69
Age at diagnosis	0.02	0.7	0.22	1.02	0.99	1.06
†Active smoker	-0.34	1.19	0.77	0.71	0.07	7.30
Extensive disease	2.72	0.72	**0.0001**	15.24	3.72	62.37
5-ASA therapy	-0.61	0.75	0.42	0.55	0.13	2.36
Azathioprine therapy	-1.04	0.77	0.18	0.35	0.08	1.59
CD (1 year outcome: surgery)						
Sex Females	1.75	1.00	0.08	5.77	0.81	41.05
Age at diagnosis	0.0009	0.02	0.68	1.01	0.96	1.06
†Active smoker	-0.07	0.81	0.93	0.93	0.19	4.59
Disease location Ileal vs. Ileocolonic and colonic	0.87	0.38	0.35	2.39	0.34	16.65
‡Disease behaviour Inflammatory vs.Penetrating/Stricturing	-2.07	0.87	**0.02**	0.13	0.02	0.70
5-ASA therapy	-1.91	1.12	0.09	0.15	0.02	1.34
Azathioprine therapy	-1.44	1.26	0.26	0.24	0.02	2.83

Table 5: The rates of corticosteroid dependence and resistance; and the use of immunosuppressants and 5-ASA therapy in this and other studies

	Current study (United Kingdom)		**Munkholm et al (Denmark)**	**Faubion et al (Minnesota, USA)**	
	UC	CD	CD	UC	CD
Number of patients	136	80	196	185	173
Started on CS	86(63%)	60(75%)	109 (56%)	63(34%)	74(43%)
At 30 days: Complete remission	51%	40%	48%	54%	58%
Partial remission	31%	35%	32%	30%	26%
No response	18%	25%	20%	16%	16%
At 1 year: Prolonged response	55%	38%	-	49%	32%
CS dependence	17%	24%	-	22%	28%
Surgery	21%	35%	-	29%	38%
Azathioprine	28%	8%	-	0%	3%
5-ASA	72%	49%	-	77%	50%

Chapter 4

Predicting the outcome of severe ulcerative colitis: Development of a novel risk score to aid early selection of patients for 2nd line medical therapy or surgery

Abstract

Background: The failure rate of medical therapy in severe UC is high. A risk index, which aids the identification of patients according to the likelihood of not responding to intravenous corticosteroid therapy, would be useful to facilitate second-line treatment or surgery.

Methods: 167 consecutive patients with severe UC between January 1995 and March 2002 were studied. I employed multiple logistic regression to analyze parameters within the first three days of medical therapy. In addition, I utilised statistical modelling to formulate a risk score according to the likelihood of medical failure.

Results: Sixty-seven (40%) patients failed to respond to medical therapy. Multivariate analysis identified mean stool frequency and colonic dilatation within the first three days and hypoalbuminaemia as independent predictors of outcome ($p < 0.001$, 0.001 and 0.002 respectively). A numerical risk score was formulated based on these variables. Patients with scores of 0-1, 2-3 and ≥ 4 had a medical therapy failure rate of 11%, 43% and 85% respectively. R.O.C. analysis of this score yielded AUC of 0.88, with a sensitivity of 85% and specificity of 75% using score ≥ 4 in predicting non-response.

Conclusion: This risk score allows the early identification of patients with severe UC who would be suitable for second-line medical therapy or surgery.

4.1 Introduction

The natural history of ulcerative colitis (UC) is variable. Whereas many patients with UC suffer symptoms that can be managed with outpatient medical therapy, a proportion experience symptoms that prove refractory to treatment, and ultimately require colectomy. Historically, 15% of patients with UC will develop a severe attack requiring intensive in-patient medical therapy. Of these, consistent data have shown that 30-40% of patients will fail to respond to medical therapy and require urgent colectomy(Jarnerot, Rolny, and Sandberg-Gertzen 1985; Buckell and Lennard-Jones 1979; Travis et al. 1996; Lindgren et al. 1998; Carbonnel et al. 2000; Chakravarty 1993). The mortality associated with such attacks has fallen from levels of 31-61% to the present level of 1-2% following the introduction of high dose corticosteroid therapy and the acceptance of a policy of early surgery in patients not responding to medical therapy(Truelove et al. 1978).

For patients who fail to respond to corticosteroids, the use of further medical therapy rather than surgery needs careful thought, bearing in mind that much of the morbidity (and mortality) of UC is associated with delayed surgery (Hyde and Jewell 1997; Lindgren et al. 1998). Therefore, the selection of patients that may benefit from second-line therapy and importantly, the timing of the administration of therapy, are central to the management of severe UC. A risk index that can stratify patients according to their respective likelihood of not responding to intravenous corticosteroid therapy early in the course of treatment will be clinically pertinent to the issues discussed above.

In the present study, I analysed early clinical parameters within a large cohort of patients presenting with acute severe UC to identify predictive factors of non-response to medical therapy. I have developed a simple objective risk score to aid the clinician in decision-making, and specifically to help select patients for either early second-line medical therapy or early surgery depending on the likelihood of non-response to standard medical therapy. The use of similar risk indices in other fields such as the Rockall score derived from similar statistical methodology, in predicting mortality in upper gastrointestinal bleeding has led to clear benefits in aiding clinical management and also the stratification of patients in further interventional trials(Rockall et al. 1996).

Although as discussed in Chapter 1, two predictive models have been described in literature, these serve primarily to identify patients for early colectomy rather than to select patients for second-line medical therapy. In a prospective study involving 49 patients with severe UC, Travis and colleagues proposed that 85% of patients with a stool frequency of >8/day or 3-8/day with C-reactive protein of 45 mg/dl after 3 days of intensive medical therapy will fail to respond and require colectomy(Travis et al. 1996). Lindgren and colleagues developed a regressional formulae to predict the likelihood of medical failure - *number of bowel movements +0.14× CRP (mg/l)>8.0,* as the optimum theoretical cut-off level to predict failure of medical therapy(Lindgren et al. 1998).

The proposed risk score developed from this cohort aimed to identify patients who are at low, intermediate and high likelihood of not responding to intensive medical therapy.

Therefore, with the improvement in medical therapy in severe UC, treatment can be targeted at patients who will benefit most from early 2nd line medical therapy.

4.2 Methods

Settings

Patients were recruited from the gastroenterology units of two university teaching hospitals (Western General Hospital and Royal Infirmary of Edinburgh) and a large district general hospital (St. John's Hospital, Livingston) in the Edinburgh and Lothian region, Scotland, covering a combined catchment area of 600 000 people.

Patients

All patients admitted for in-patient management of acute UC between January 1995 and March 2002 were identified using the regional database of medical/surgical admissions (Lothian Surgical Audit) and respective local hospital discharge databases. Ethical approval was obtained from the Lothian Research and Ethics Committee and informed consent was obtained from each patient involved.

Case definitions

The diagnosis of UC was confirmed on clinical, radiological and histological criteria (Lennard-Jones 1989). A severe episode of UC was defined according to the modified Truelove and Witts criteria as, ≥6 episodes of bloody diarrhoea/24 hour with one or more of the following features: anemia (hemoglobin < 10.5g/dl), fever (>37.8°C), tachycardia

(pulse >90/min) or elevated ESR (> 30mm/hour). Colonic dilatation was defined by the diameter of the transverse colon on plain abdominal x-ray, ≥5.5 cm.

Data collection

For each patient, all clinical data recorded during hospital admission were retrieved from case note review. Fifty-six variables within the first three days of medical therapy were recorded. These included demographic data, clinical observations, laboratory parameters, X-ray and endoscopic assessments of the severity of UC.

Outcomes

The primary outcome was categorized as response (no colectomy) or non-response to medical therapy (colectomy) within the period of hospitalisation. Statistical modelling was also performed on secondary outcomes such as colectomy at 60 days following presentation (to account for patients who have undergone a semi-elective colectomy or those with an early severe relapse); and in the setting where intravenous ciclosporin was considered as failure of 1st line medical therapy (to account for the confounding effect of second-line medical therapy).

Statistical Analysis

Univariate analyses were initially performed to identify potential determinants of outcome of all 56 variables recorded. Of these, twenty-four potential variables were appropriate for further analysis following the removal of those variables with no prognostic significance (Appendix 1). Forward stepwise multiple logistic regression was

then employed to identify predictive factors (with $\alpha>0.1$ taken as a level of exclusion) associated with our outcomes. To generate a clinical predictive model, variables of prognostic significance were categorized and re-entered into a logistic regression model. An integer score was attributed to each category of each variable according to its relative contribution in the regression model (as determined by its regression coefficient in the logistic equation). The scores were then grouped to provide a practical triage into low, intermediate and high risk categories. All analyses were performed using the Minitab statistical software.

4.3 Results

1211 admissions with inflammatory bowel disease were identified between January 1995 and March 2002. Comprehensive review of these cases led to the identification of 245 patients admitted to hospital with acute flare of ulcerative colitis, of whom, 167 (68%) patients fulfilled the criteria of Truelove and Witts. Sixty-eight patients (40%) failed to respond to medical therapy and required colectomy during that admission. The median time to colectomy was 9 days following admission (interquartile range, IQR- 7 to 15 days) (Figure 1). Two of these patients died during the period of hospitalisation (post-operative pneumonia and arterial thrombosis of lower limb respectively). The median duration of admission for non-responders and responders was 26 and 11 days respectively.

Patient details (Table 1)

The median age at presentation was 38 years (IQR – 27-54). Males accounted for 61% of the patient population. The median duration of relapse prior to admission was 4 weeks (IQR 2-9.5 weeks). Seventy-six (45%) patients were experiencing the first attack of UC, 91 patients (55%) were known to have UC; and 42 (25%) patients had previous admissions with severe colitis. Eight (5%) patients were current smokers. Extensive colitis (beyond the splenic flexure) was present in 131 (81%) patients. Of these, 62(47%) patients with extensive colitis were non-responders. In comparison, only 10% of patients with disease limited to recto-sigmoid area failed to respond to medical therapy ($p<0.001$).

Management (Table 2)

All patients were treated with standard medical therapy of intravenous corticosteroids (methylprednisolone 60 mg/day or hydrocortisone 400mg/day). In addition, 83% of patients received oral 5-aminosalicylate (5-ASA) preparations, 45% received concurrent topical therapy (rectal 5-ASA or corticosteroids), 71% received subcutaneous heparin for thrombo-embolic prophylaxis, and 13% received intravenous ciclosporin and total parenteral nutrition.

Twenty-one patients received intravenous cyclosporin at dosage of 4mg/kg. The median time for cyclosporin therapy was 10 days after the initiation of intravenous corticosteroids. This however, was shorter in the non-responders (7 days vs. 12 days). No patients had evidence of colonic dilatation. Nine patients responded, with the remainder requiring colectomy within the period of hospitalization ($p=0.089$). Of those that

responded, 3 patients subsequently required colectomy during follow-up (median time to colectomy – 2.4 years, range 1.4-3.2 years).

Of the twenty-one (13%) patients who had received intravenous ciclosporin (4mg/kg), only 9 patients avoided colectomy during the period of hospitalisation. Of those that responded, 3 patients subsequently required colectomy during follow-up (median time to colectomy – 2.4 years, range 1.4-3.2 years). The median time for the initiation of cyclosporin therapy was shorter in the non-responders (7 days vs. 12 days).

Univariate analysis of clinical and laboratory parameters (Table 2)

A significantly higher percentage of non-responders presented with stool frequency >8/day on admission (58.8% vs. 29.3%, p<0.001, OR-0.29, 95% CI 0.15-0.56). On each of days 1 to 3, there was a consistently maintained difference in stool frequency between responders and non-responders (data not shown), and we compared the discrimination of the mean stool frequency over the first three days with data of each individual day. This derived variable showed a more significant difference between responders and non-responders than the stool frequencies on any individual days.

Evidence of hypoalbuminaemia (<30g/l) was present in 47% and 28% of non- and responders respectively on admission (p=0.005). Serum albumin was also noted to be significantly lower on day one of therapy in non-responders (mean g/l ±SD) 30.6g/l ±5 compared with responders, 34.1g/l ±6 (p=0.001, OR-1.10, 95% CI 1.04-1.17). Similarly, C-reactive protein levels on day one of medical therapy (median mg/l± IQR) were also

significantly higher in non-responders (6.9 mg/l, IQR 2.8-19.2 compared with responders, 3.9 mg/l, IQR 1.5-9.3 (p =0.02). Colonic dilatation was present in 16 patients (9.6%) of these, 15 patients required colectomy (p=0.001, OR-0.04, 95% CI 0.00-0.29). Weaker trends were observed in platelet count and ESR.

Other comparisons, in particular, the proportions of patients experiencing their first attack, the duration of relapse prior to hospitalisation and clinical parameters such as neutrophil count, temperature, pulse rates and stool consistency were not associated with outcome.

Multivariate analysis and modelling (Table 2)

Mean stool frequency (MSF), the presence of colonic dilatation within the first three days and serum albumin on day 1 of medical therapy were identified as significant independent predictors of outcome in our cohort (p <0.001, <0.001 and 0.002 respectively). Notably, C-reactive protein, platelet and ESR measurements implicated in the univariate analysis did not achieve statistical significance in multivariate analysis.

As significant differences were also observed on univariate analysis of the additional medical therapies (Table 2), multivariate analysis was carried out separately to assess the effect of these therapies on the outcomes of our patients. When considered together with the 24 parameters originally included in the earlier multivariate analysis, only total parenteral nutrition demonstrated significant association with non-response to medical

therapy. It is noteworthy that 5-ASA and subcutaneous heparin which were implicated on univariate analyses did not achieve statistical significance. TPN however, was not considered in statistical modelling as the median time to the commencement of this treatment was 6 days (IQR 4-7 days) following the initiation of intravenous corticosteroid therapy.

In order to generate a scoring system, MSF and serum albumin values were categorized using integer cut-points guided by the receiver operator characteristic (ROC) curve and observed relationship with outcomes.

This resulted in the categorization of MSF as:

- ≤ 4 stools/ 24 hours
- > 4 stools but ≤ 6 stools/ 24 hours
- > 6 stools but ≤ 9 stools/ 24 hours
- > 9 stools / 24 hours

And serum albumin as:

- ≤ 30 g/l
- > 30 g/l

Colonic dilatation was regarded as present or not present within the first three days of therapy.

To formulate a numerical risk score, we used the coefficients generated by the logistic regression equation, to derive an integer number, approximating the values of the coefficients for each of the categories above (Table 2).

Other models (Table 4)

I also explored different settings for modelling. These were;

A. Analyses of outcomes at 60 days to account for early severe relapse or semi-elective surgery in partial responders.
B. Analyses where ciclosporin treatment was regarded as failures of medical treatment.
C. Analyses where colonic dilatation was excluded as some centers may regard this sign as an absolute indication for surgery.

In model A, a further 9 patients required colectomy within 60 days (3 partial and 6 complete responders). In model B, 9 patients who had received ciclosporin (but no colectomy during admission) were regarded as primary treatment failure. For model C, 16 patients with colonic dilatation were excluded. Multiple logistic regression analysis failed to identify prognostic factors additional to stool frequency, serum albumin and colonic dilatation.

Final model

Using the coefficients of the regression analyses, the MSF of ≤ 4 stools/ 24 hours, >4 and ≤6 stools/ 24 hour, >6 and ≤9 stools and > 9 stools/ 24 hours were given scores of 0, 1, 2 and 4 respectively. The presence of colonic dilatation was attributed a score of 4 (or score

0, in the absence of this sign) and hypoalbuminaemia (≤30g/l), score of 1 (or score 0, albumin >30g/l) (Table 5). Using the scoring system, each patient in our study was scored and compared to his/her eventual outcomes.

For an individual patient, the total score was derived from the sum of the score attributed to MSF (0-4), colonic dilatation (0 or 4) and serum albumin (0 or 1). The minimum score possible (0) would be attributed to a patient with MSF<4, no evidence of colonic dilatation or hypoalbuminaemia and a maximum score of 9, to a patient with MSF>9 (4 points) together with the presence of both colonic dilatation (4 points) and hypoalbuminaemia (1 point).

The rates of non-response according to prognostic scores are demonstrated in Figure 2. All patients with scores of ≥6 failed to respond to medical therapy (3 patients scoring 6, 6 patients scoring 7 and one patient scoring the maximum value of 9). Proportions of patients falling into each score categories were 19%, 23%, 9%, 25%, 13%, 5% and 5% respectively.

It is possible to triage patients to the groups of low (score 0-1), intermediate (score 2-3) and high (score ≥4) risk groups with medical failure rates of 11%, 45% and 85% respectively. Receiver-operating characteristic (R.O.C) analysis for this risk score yielded an area under the curve (A.U.C) of 0.876 (AUC of 1.0 indicating perfect test) (Figure 3). The sensitivity and specificity for predicting non-response to medical therapy with scores of ≥4 was 85% and 75% respectively. The AUC of this current model for colectomy at 60

days following presentation, ciclosporin treatment was regarded as primary treatment failure and where colonic dilatation were excluded were 0.833, 0.810 and 0.807 respectively.

4.4 Discussion

The present analysis involves the most recent and largest cohort of patients presenting with severe UC reported in detail. In a 7-year period, I reported on 167 consecutive patients with acute severe UC satisfying the modified Truelove and Witts criteria. Consistent with previous studies (Travis et al. 1996; Buckell and Lennard-Jones 1979; Carbonnel et al. 2000; Jarnerot, Rolny, and Sandberg-Gertzen 1985; Lennard-Jones et al. 1975; Chakravarty 1993), the failure rate of medical therapy is high (40%). On multivariate analysis, mean stool frequency and colonic dilatation within the first three days of therapy and hypoalbuminaemia on day 1 of treatment, were significantly predictive of the need for surgery within the period of hospitalisation ($p<0.001$, <0.001 and 0.002 respectively). Using statistical modelling, I have formulated a numerical risk score that assesses the individual's likelihood of non-response. This risk score enables the stratification of our patients to those with low (11%), intermediate (45%) and high risks (85%) of not responding to therapy within 3 days of medical therapy.

Of greatest clinical relevance is the identification of low and high risk groups in which surgery was either very unlikely or likely. I propose that in the intermediate group (34% of patients), close monitoring is essential with the option of second-line medical therapy at an early stage. This benefit may also apply to the high risk group; however, a delay in surgery in this group may lead to an unacceptable increase in the risk of complications,

and the case for early surgery (rather than further medical intervention) may be more compelling in patients with high risk score.

Different clinically relevant settings were considered for statistical modelling – colectomy at 60 days to account for early severe relapse or semi-elective surgery for partial responders with chronic continuous symptoms, where second-line therapy such as ciclosporin was considered as primary treatment failure and where colonic dilatation was excluded from analysis. Nevertheless, in this cohort, no further factors were identified from multivariate analysis. I have accounted for colonic dilatation in statistical modelling, on the basis that some may consider this finding as an indication for surgery by itself. Indeed, approximately 10% of patients in the current cohort had radiological evidence of this within the first 3 days of hospitalization.

C-reactive protein concentration was a notable exclusion from the multivariate logistic regression analysis. The explanation for this bears evaluation but it is conceivable that in a pre-selected group with unequivocal severe disease, the absolute level of acute phase reactants may not be as useful as the change in response to intensive medical therapy. Differences observed in 5-ASA, total parenteral nutrition and heparin therapy in univariate analyses were thought be due to confounding effect (e.g. physician-led reaction to illness and malnutrition).

Is this risk score relevant to clinical practice? The sensitivity (85%) and specificity (75%) of this risk score in predicting non-response to medical therapy at the cut-off point of ≥ 4

compared favourably with two other models described in the literature(Travis et al. 1996; Lindgren et al. 1998). The sensitivity/specificity for the models described by Travis and Lindgren et al. when applied to our data, were 58%/71% and 61%/88% respectively (data not shown). This maybe explained by the differences in the severity of illness and populations, of the cohorts studied in Oxford, Stockholm and Edinburgh, indeed; the two previous populations did not display significant hypoalbuminaemia and colonic dilatation that were evident in 40% and 10% respectively in our cohort. This is supported by the higher colectomy rate observed in our cohort (40% vs. 31%/34% in the cohorts of Travis and Lindgren respectively).

The ability to categorize patients on day 3 allows the use of potentially toxic second-line therapy or early surgery targeted to the individual patient according to risk. A recent UK-wide multi-centre survey conducted by the British Society-IBD network showed that even in an unselected cohort of patients receiving in-patient medical therapy for acute UC, 28% of patients required colectomy as a consequence of failed medical therapy. Consistent with the present data, the usage of intravenous ciclosporin therapy was low (13%).

Although, the use of second-line medical therapy is complicated by other issues such as patient choice, familiarity and experience of the treating centre; there is robust data regarding efficacy and safety of ciclosporin therapy. Van Assche et al. most recently demonstrated the safety and efficacy (85% response rate) of ciclosporin therapy at 2mg/kg dosage regime. Short term colectomy rate was 8.6%(Van Assche et al. 2003).

Along with the recent report of favourable long term response in initial responders to ciclosporin subsequently maintained on Azathioprine therapy(Cohen, Stein, and Hanauer 1999), quality of life following successful ciclosporin therapy is comparable if not better than patients who have underwent surgery(Cohen RD, Brodsky AL, and Hanauer 1999). More recently, infliximab as discussed in Chapter 1, has been shown to be useful in acute severe UC(Jarnerot et al. 2005b). Therefore, the use of second-line medical therapies is increasing and this risk index provides the guidance in identifying and counselling patients, for clinicians on a wider scale.

The primary development of this risk score therefore, has important implications in allowing targeted therapy aimed at the group of patients that will benefit most from early second-line medical therapy, selection of patients for early surgery, patient counselling and the stratification of patients according to risk, in future therapeutic trials. These, we believe, will improve the clinical management of severe UC. This risk model precisely addresses the positioning of biological therapies such as infliximab in acute severe UC, with the patients in intermediate and high risk groups in particular benefiting from early therapy (Figure 3).

Table 1: Univariate analysis of parameters in 167 patients presenting with acute severe UC. [1] The duration of relapse was the time of the onset of symptoms to hospital admission. [2]The disease extent was based on both colonoscopic and histological features. Subtotal disease extends beyond the splenic flexure. [3] Patients admitted with a first acute severe UC episode. *-Truelove and Witts criteria (stool frequency>8/day and/or temperature >37.8°C, pulse >90/minute, haemoglobin, 10.5g/dl and ESR >30mm/hour). Mean values of the first three days of therapy. *-Colonic dilatation - transverse colon diameter >5.5cm. [§] Median values of CRP shown with interquartile range. †- Laboratory parameters were based on values from day 1 of medical therapy. For continuous variables in laboratory parameters, odds ratio calculated per unit of change. [n] -Odds ratio for platelets calculated per change of 10 units.

	Overall (n=167)	**Non-responders** (n=68)	**Responders** (n=99)	**Odds ratio** (95% CI)	**P-value**
Age (years, IQR)	38 (27-54)	44 (28-57)	37 (27-50)	-	0.07
Sex (M:F)	103:64	47:21	56:43	-	0.10
Duration of relapse[1] (weeks, IQR)	4 (2-9.5)	4 (2-9)	4 (2-9)	-	0.61
Disease extent[2]					
Recto-sigmoid	31 (19%)	3 (5%)	28 (28%)		<0.001
Subtotal/Total	131 (81%)	62 (92%)	69 (69%)		
First presentation[3]	76 (45%)	29 (43%)	47 (47%)	-	0.13
Current smoker	8 (5%)	2 (3%)	6 (6%)	-	0.53
Previous admissions (requiring IV corticosteroids)	42 (25%)	19 (28%)	23 (23%)	-	0.49
Clinical observation Admission (SD)					
Stool frequency >8/day	69 (41.3%)	40 (58.8%)	29 (29.3%)	0.29 (0.15-0.56)	<0.001
Temperature (°C)	37.4 (0.7)	37.4 (0.8)	37.3(0.7)	-	0.51
Pulse (/min)	90.9 (14.3)	90.5 (13.4)	91.3 (15.5)	-	0.73
Stool consistency-Fluid (%)	140 (84%)	56 (84%)	84 (85%)	-	0.38

Mean (Day 1-3)(SD)					
Stool frequency	6.34 (3.2)	8.05 (3.4)	5.2 (2.4)	0.71 (0.62-0.81)	<0.001
Pulse	85.5 (12.0)	86.4 (13)	84 (11.2)	-	0.42
Temperature	37.07 (0.48)	37.16 (0.52)	37.00 (0.45)	0.51 (0.26-0.98)	0.04
Colonic dilatation	16 (9.6%)	15 (22%)	1 (1%)	0.04 (0.00-0.29)	<0.001
Drugs prior to admission					
5-ASA (800-1200mg/day)	91 (56%)	37 (57%)	54 (54%)	-	0.93
Oral Prednisolone (20-40mg/day)	75 (46%)	34 (52%)	41 (41%)	-	0.24
Topical therapy	35(22%)	10 (15%)	25 (25%)	-	0.11
Azathioprine (2mg/kg/day)	13(8%)	4 (6%)	9 (9%)	-	0.46
In-patient drug therapy					
5-ASA (800-1200mg/day)	139 (83%)	50 (74%)	89 (89%)	3.14 (1.35-7.32)	0.008
S.C. heparin (5000U/day)	118 (71%)	60 (88%)	58 (58%)	0.19 (0.08-0.43)	<0.001
Ciclosporin (4mg/kg)	21 (13%)	12 (18%)	8 (8%)	-	0.06
Total Parenteral Nutrition	22 (13%)	19 (28%)	2 (2%)	0.08 (0.02-0.29)	0.004
Topical therapy	75 (45%)	25 (37%)	50 (50%)	-	0.09
Laboratory parameters†(SD)					
Haemoglobin (g/l)	12.0 (2.3)	11.7 (2.3)	12.3 (2.2)	-	0.09
Neutrophil ($x10^9$)	9.7 (3.7)	9.8 (4.4)	8.9 (3.4)	-	0.18
Platelet ($x10^9$)[n]	426.3 (148.7)	461.0 (164.0)	402.5 (133.0)	0.97 (0.95-0.99)	0.01
ESR (mm/hr)	44.8 (26.5)	50.5 (28.9)	41.0 (24.2)	0.99 (0.97-1.0)	0.04
CRP (mg/l)[§]	4.4 (2.1-13.3)	6.9 (2.8-19.25)	3.9 (1.5-9.35)	0.98 (0.95-1.01)	0.02
Albumin (g/l)	32.7 (6.2)	30.6 (5.0)	34.1 (6.2)	1.10 (1.04-1.17)	0.001

(Continued from Table 1).

Table 2a: Multiple logistic regression analysis of clinical parameters within first three days of medical therapy identified mean stool frequency (MSF), the presence of colonic dilatation and day 1 serum albumin as significant predictors of failure of medical therapy.

Variables	Coefficient	SE Coefficient	P-value	Odds ratio	95% CI	
					Lower	Upper
MSF	-0.378	0.06	<0.001	0.68	0.61	0.78
Colonic dilatation	-3.548	1.11	0.001	0.03	0.00	0.20
Day 1 serum albumin	0.09	0.03	0.002	1.10	1.03	1.15

Table 2b: MSF and serum albumin were grouped into the categories above using ROC curve analysis and adapted according to clinical relevance. These categories were re-entered into the logistic regression model. MSF <4/day was used as reference index in MSF category. Formulation of integer risk score for each category was based on the strength of contribution to logistic equation based on the coefficient (*For example- The coefficient of MSF>9 is -4.27, therefore an integer score of 4 was given, coefficient of colonic dilatation is -3.8, therefore an integer score of 4 is given).* MSF-mean stool frequency.

Variables	Coefficient	SE Coefficient	P-value	Odds ratio	95% CI	
					Lower	Upper
Constant	-	-	-	-	-	-
MSF 4-< 6/day	-1.40	0.73	0.055	0.25	0.06	1.03
Stool 6-< 9/ day	-2.20	0.69	0.002	0.11	0.03	0.43
Stool > 9/day	-4.3	0.84	<0.001	0.01	0.00	0.07
Colonic dilatation	-3.8	1.17	0.001	0.02	0.00	0.22
Serum albumin < 30g/l	-1.24	0.44	0.005	0.29	0.12	0.69

Table 3: Multiple logistic regression of clinical parameters within 1st three days of medical therapy and medical therapy (5-ASA, topical therapy, subcutaneous heparin, total parenteral nutrition –TPN, ciclosporin). Only TPN was shown to be significant in predicting outcome with MSF, colonic dilatation and day 1 albumin.

Variables	Coefficient	SE Coefficient	P-value	Odds ratio	95% CI	
					Lower	Upper
MSF	-0.435	0.08	<0.001	0.65	0.55	0.76
Colonic dilatation	-3.489	1.15	0.003	0.03	0.00	0.29
Day 1 serum albumin	0.09	0.04	0.025	1.10	1.01	1.19
TPN	-2.968	0.93	0.001	0.05	0.01	0.32

Table 4: Summary of multiple logistic analyses for 3 alternative models. Model A – analysis based on outcome of surgery vs. no surgery at 60 days following discharge; Model B- analysis where treatment with IV ciclosporin was regarded as failure of medical treatment in addition to surgery within the period of hospitalisation.; Model C - analysis based on outcome of surgery vs. no surgery within the period of hospitalisation with colonic dilatation excluded. Using these models described above, no additional prognostic factors were identified. Odds ratio of MSF, serum albumin and colonic dilatation of the final model were compared with models A, B and C. †-All patients with colonic dilatation either required colectomy or received ciclosporin therapy. ‡MSF-mean stool frequency of the first three days of therapy.

	Outcome (within hospitalisation)		**Model A**		**Model B**		**Model C**	
Variables	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
MSF‡	0.66 (0.58-0.78)	<0.001	0.68 (0.59-0.77)	<0.001	0.69 (0.59-0.79)	<0.001	0.69 (0.61-0.79)	<0.001
Serum albumin	1.11 (1.03-1.16)	0.002	1.10 (1.02-1.15)	0.005	1.12 (1.03-1.15)	0.001	1.10 (1.03-1.16)	0.002
Colonic dilatation	0.02 (0.00-0.16)	<0.001	0.04 (0.004-0.35)	<0.001	∞ †	<0.001	Excluded	-

Table 5: Integer risk score attributable to each category derived from the coefficients of the logistic regression equation. Overall risk core = [score attributable to mean stool frequency (0, 1, 2 or 4)] + [presence of colonic dilatation (0 or 4)] + [presence of hypoalbuminaemia (0 or 1)]. Minimum score = 0, maximum score = 9.

Variables	**Score**
Mean stool frequency	
<4	0
4-<6	1
6-<9	2
>9	4
Colonic dilatation	4
Hypoalbuminaemia	
<30g/l	1

Figure 1: Outcome of 167 consecutive patients with severe UC.

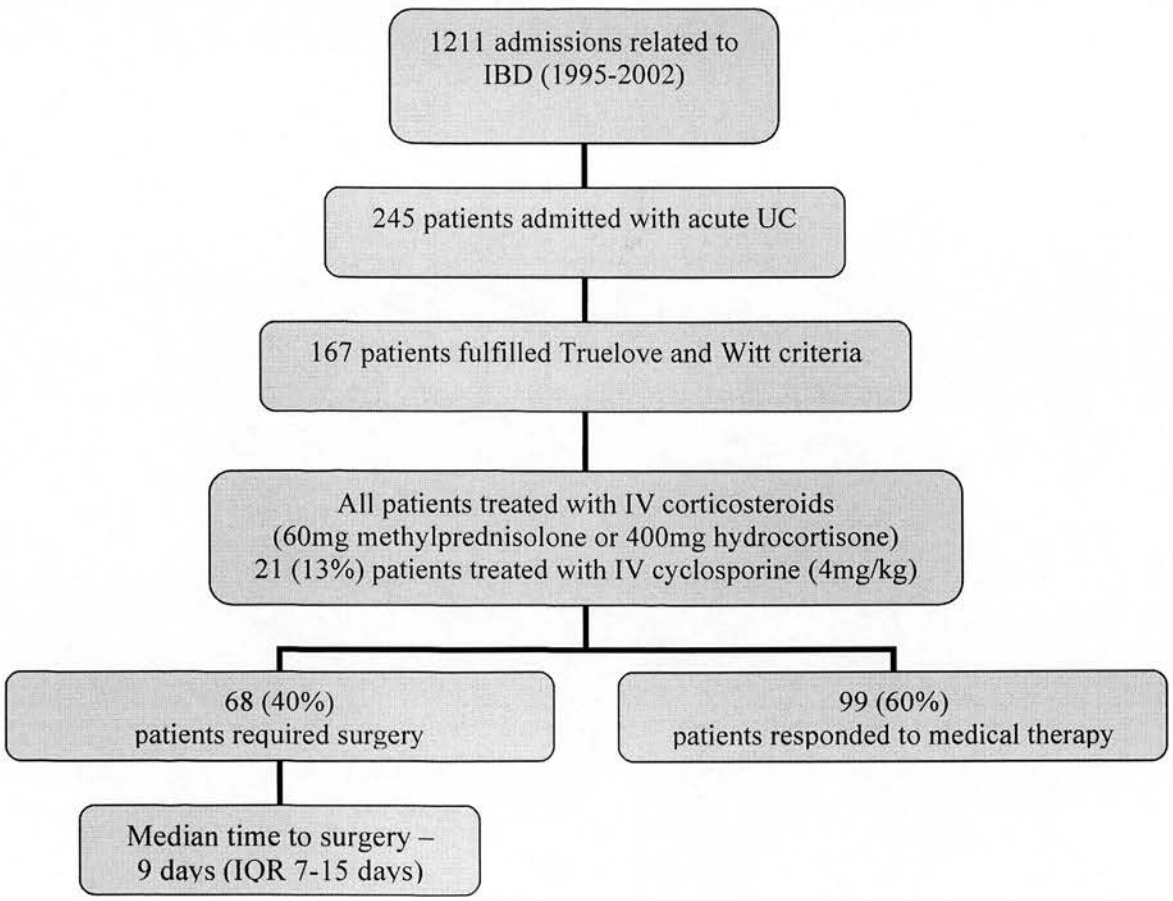


Figure 2: Rate of failure of medical therapy according to numerical risk score in 167 patients presented with acute severe UC.

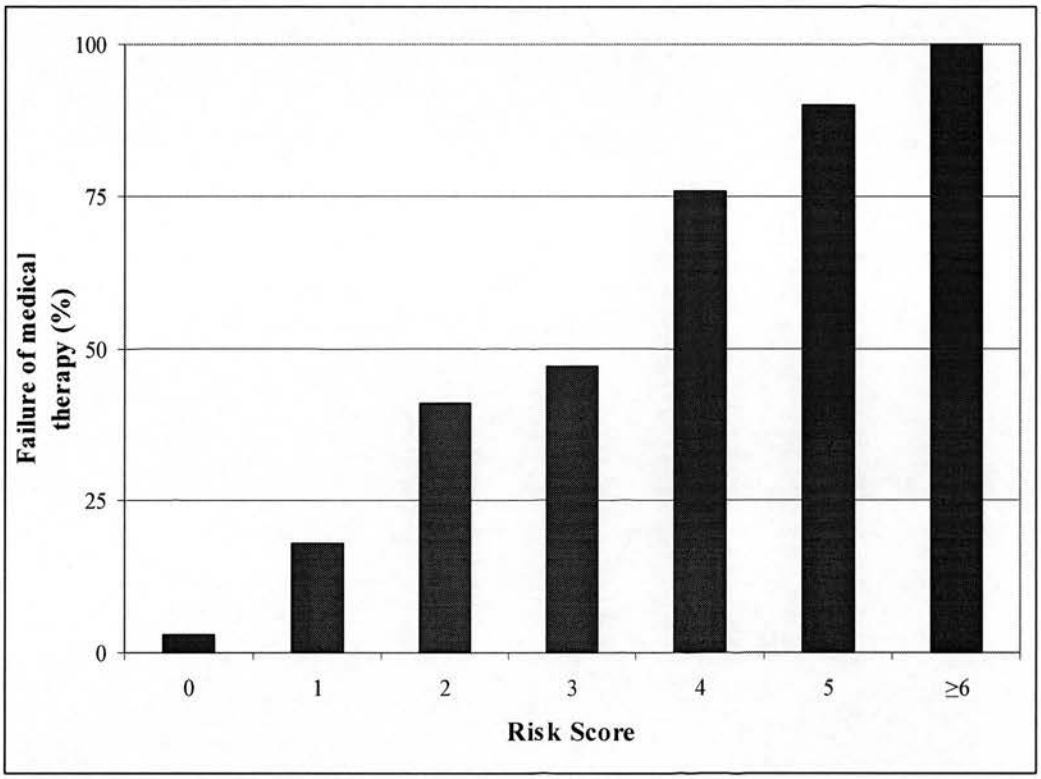


Figure 3: Receiver-operator characteristic (ROC) curve of risk score, yielding an area under curve (AUC) of 0.876. The AUC of this current model for colectomy at 60 days following presentation, cyclosporine treatment was regarded as primary treatment failure and where colonic dilatation were excluded were 0.833, 0.810 and 0.807 respectively.

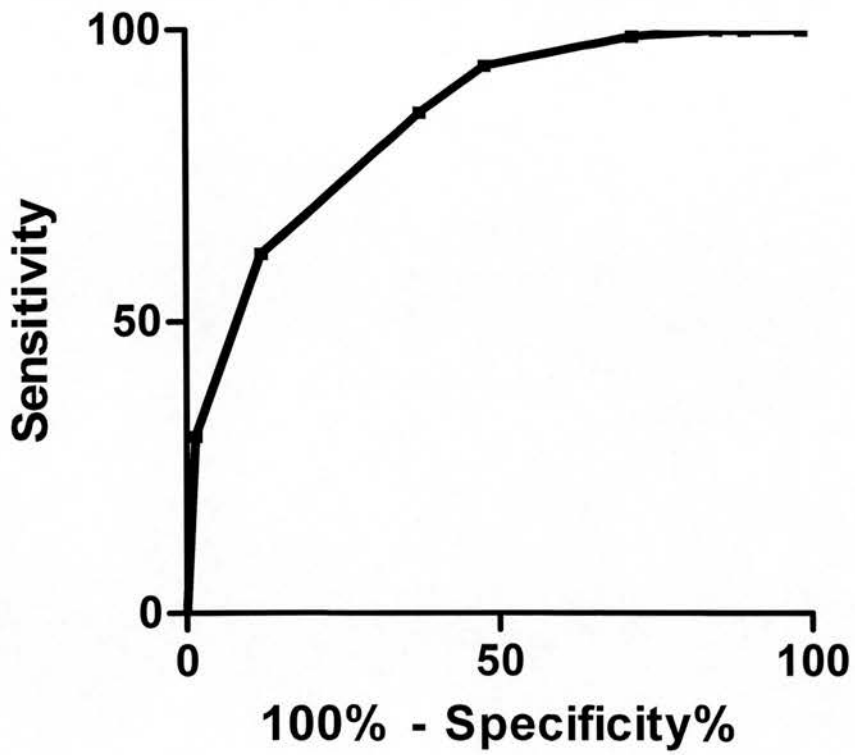
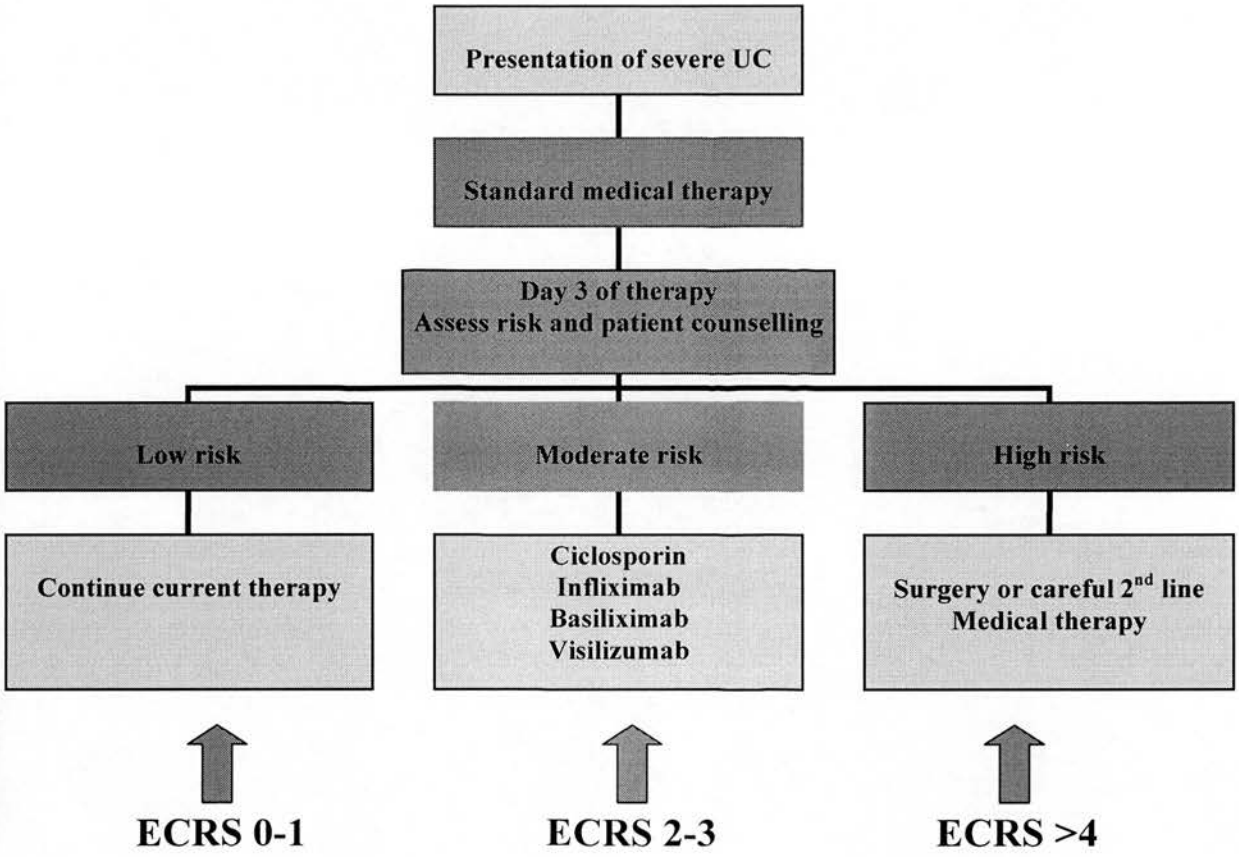


Figure 3: The new model of management of severe ulcerative colitis.



Chapter 5

Allelic variations of the multidrug resistance gene (MDR1) determine susceptibility and disease behaviour in ulcerative colitis

Abstract

Background/Aims: The MDR1 gene encodes P-glycoprotein 170, an efflux transporter which is highly expressed in intestinal epithelial cells. The MDR1 exonic SNPs C3435T and G2677T have been shown to correlate with activity/expression of P-glycoprotein 170.

Methods: Case-control analysis of MDR1 C3435T and G2677T SNPs in a large well-characterized Scottish-Caucasian cohort (335 UC, 268 CD and 370 healthy controls). 2-locus haplotype and detailed univariate and multivariate genotypic-phenotypic analyses were performed.

Results: The MDR1 3435 TT-genotype (34.6% vs. 26.5%, p=0.04, OR 1.60, 95% CI 1.04-2.44) and T-allelic frequencies (58.2% vs. 52.8%, p=0.02, OR 1.28 95% CI 1.03-1.58) were significantly higher in patients with UC compared to controls. No association was seen with CD. The association was strongest with extensive UC (TT-genotype, 42.4% vs. 26.5%, p=0.003, OR 2.64 95% CI 1.34-4.99 and T-allele, 63.9% vs. 52.8%, p=0.009, OR 1.70 95% CI 1.24-2.29), and this was also confirmed on multivariate analysis (p=0.007). The G2677T SNP was not associated with UC or CD. These 2 SNPs lie in linkage disequilibrium in our population (D' 0.8-0.9 and r^2 0.7-08). 2-locus haplotypes showed both positive (3435T/G2677 haplotype; p=0.03, OR 1.44) and negative (C3435/2677T haplotype, p=0.002, OR 0.35) associations with UC. Homozygotes for the haplotype 3435T/G2677 were significantly increased in UC (p=0.017, OR 8.88 95% CI 1.10-71.45).

Conclusion: Allelic variations of the MDR1 gene determine disease extent as well as susceptibility to UC in the Scottish population. The present data strongly implicate the C3435T SNP, although the 2-locus haplotype data underline the need for further detailed haplotypic studies.

5.1 Introduction

Recent attention has focussed on the multidrug resistance 1 (MDR1) gene and its product, the P-glycoprotein 170 as a potential determinant of susceptibility to IBD(Ho, Moodie, and Satsangi 2003). P-glycoprotein 170, which functions as an ATP-dependent efflux transporter pump is highly expressed in the epithelial surfaces of intestine, biliary ductules, proximal tubules of kidneys and central nervous system where it forms the basis of the blood-brain barrier(Thiebaut et al. 1987; Cordon-Cardo et al. 1989; Fojo et al. 1987). Interindividual variability of P-glycoprotein expression in the intestine plays a role in the determining the pharmacokinetics of wide-ranging number of substrates. Nevertheless, the exact physiological role in the gut remains unknown. The high constitutive levels of expression of P-glycoprotein 170 in the gut suggest a role in protection not only against xenobiotics but bacterial products.

As reviewed in depth in chapter 1, MDR1 gene is an attractive candidate gene for IBD for several reasons. Firstly, mdr-1a deficient mice develop a UC-like phenotype when maintained in specific pathogen free environment which is reversed by antibiotics(Panwala, Jones, and Viney 1998). Bone marrow transfer studies show that these mice develop colitis primarily due to deficiency of mdr-1 in the epithelial rather than the lymphoid cells. Secondly, MDR-1 gene maps to chromosome 7q22 which has been identified as a putative locus of susceptibility for IBD, by genome-wide scanning in a UK cohort. Recent subsequent meta-analysis of all genome-wide scans confirms suggestive linkage to this region (van Heel et al. 2004). Most recently, compelling data by Langmann et al. have demonstrated that the MDR1 gene expression is downregulated

in IBD with expression significantly reduced in the colonic tissue of patients with UC but not CD (Langmann et al. 2004).

The MDR1 gene is composed of 28 exons and is 209 kb in length(Hoffmeyer et al. 2000b; Cascorbi et al. 2001b; Kim et al. 2001b). Two SNPs, exonic variant C3435T and G2677T/A have been shown to correlate with activity/expression of P-glycoprotein 170. The C3435T SNP in exon 26 has been most extensively investigated and was first shown to correlate with expression of P-glycoprotein 170 (Hoffmeyer et al. 2000b). In this study, the TT genotype was associated with lowered intestinal P-glycoprotein 170 expression with functional consequence as inferred by increased digoxin uptake following oral administration(Cascorbi et al. 2001a). This has been replicated in other pharmacokinetic studies(Hitzl et al. 2001; Kim et al. 2001a; Johne et al. 2002b). In addition, the effect of the MDR1 C3435T SNP and its postulated correlation with P-glycoprotein 170 activity/expression has been shown to play a role in drug-resistant epilepsy (Siddiqui et al. 2003), immune recovery after initiation of anti-retroviral therapy in HIV (Fellay et al. 2002) and the development of renal cell carcinoma(Siegsmund et al. 2002). The G2677T/A SNP in exon 21 results in 2 distinct amino acid changes namely, 893Ser (G2677T) or the much rarer, 893Thr (G2677A); and has been shown to be associated with altered transporter function or expression (Kim et al. 2001a; Tanabe et al. 2001). Several studies have suggested that the C3435T and G2677T SNP may lie in linkage disequilibrium(Johne et al. 2002a; Kim et al. 2001a; Zheng et al. 2002; Horinouchi et al. 2002).

I aimed firstly to investigate the contribution of the MDR1 C3435T and G2677T SNP in a large independent well-characterized population of Scottish-Caucasians. I then determined whether 2-locus haplotypes provide a stronger association with disease than individual SNPs, in order to resolve the controversies regarding the contribution of this gene to disease susceptibility. In addition to this, I rigorously conducted a detailed sub-phenotypic review of all subjects genotyped to assess whether these variants are particularly implicated in determining disease extent and behaviour in IBD. In order to clarify whether MDR1 genotypes may influence drug responsiveness in UC, I further subcategorised the patients to the phenotypes of severe disease and need for surgery in UC.

5.2 Methods

Patients

This study was approved by the Lothian Research and Ethics Committee (LREC) and written consent was obtained from all patients. A total of 335 patients with UC and 268 with CD were recruited from the Lothian region, Scotland, United Kingdom. The diagnosis of IBD was determined by standard clinical, radiological, endoscopic and histological criteria. Table 1 summarizes the clinical characteristics of patients studied. The median ages of diagnosis for UC and CD were 35.0 years (IQR 25.3-50.3) and 26.6 years (IQR 19.9-37.0) respectively. The ethnicity of this study population was predominantly Scottish-Caucasian (99%). There were more males in the UC cohort (54.5%) than in CD (43.5%).

Phenotypic assessment

UC phenotype was classified by disease extent, severity and need for surgery. The ***extent of disease*** was documented at time of latest follow-up. Extensive disease was defined as disease extending beyond splenic flexure, left-sided colitis as disease extending to the splenic flexure and proctitis, limited to the rectum as determined by histological and macroscopic evidence. In discordant cases, the histological evidence was used. Patients who had developed acute severe attack of UC (satisfying the Truelove and Witts criteria) requiring intensive in-patient medical therapy were regarded to have ***severe UC***. Within the severe UC, a further subset of patients who had failed to respond to medical therapy and consequently required surgery were categorized under the phenotype – ***need for surgery***. Other phenotypic details such as smoking, family history, presence of primary sclerosing cholangitis (PSC) and other extra-intestinal manifestations were also recorded.

CD was classified according to the Vienna Classification of disease location, behaviour and age of diagnosis, as previously described (Gasche et al. 2000). We have previously shown that disease behaviour is not stable over time, and therefore have analyzed disease behaviour only at time of diagnosis and at latest follow-up (Smith et al. 2004).

Controls

370 healthy controls which comprised of actively recruited healthy subjects (n=105) and blood donors (n=265) were all recruited from the Lothian region between 2000-2002. There were no differences in the demographics between these 2 groups. There overall

gender distribution was 179 males:191 females; median age of recruitment was 37.1 years (IQR 25.9-47.0).

Genotyping

Genotyping was performed using TaqMan (ABI, San Diego, CA). TaqMan probes were available from ABI-assay-on-demand/design – C3435T (rs1045642) and G2677T/A (rs2032582). Sequence and reactions were described in Chapter 2.

The G2677T/A is a tri-allelic SNP, with reported frequencies of the rare A-allele in European Caucasian ranging from undetected to 4% (Furuno et al. 2002; Cascorbi et al. 2001b; Kim et al. 2001a; Gaikovitch et al. 2003) (Gerloff et al. 2002). I have sequenced 200 chromosomes of the UC group (100 individuals) and confirmed a G2677A-allelic frequency of only 2% in our population. In view of this low frequency, I have chosen to genotype the 2 common variants of the G2677T/A SNP, using TaqMan reaction.

Statistical analysis

Genotype and allelic frequencies between cases and controls were compared using a 2X2 table and Fisher's exact test. Odd ratios (OR) were given with 95% confidence intervals and 2-sided p-values. A p-value of ≤ 0.05 was considered significant. All calculations were performed using the Graph Pad Instat programme (Graph Pad Software, San Diego, USA). Fisher's exact test was utilized to evaluate if the homozygote and heterozygote frequencies for each SNPs deviate from Hardy-Weinberg equilibrium. 2-locus haplotypes frequencies were measured using the the expectation-maximization algorithm utilizing

the SNPHAP programme (bioinformatic programmes available and accessed via the Medical Research Council-Rosalind Franklind Centre of Genomic Research website: www.rfcgr.mrc.ac.uk)(Zhao, Curtis, and Sham 2000).

Multivariate analysis was performed using a logistic regression model to test the association between phenotype and genotypes. Two methods were used for haplotypic association with disease, and these were: log-likelihood ratio method where inferred haplotypes were compared in case, control and case/controls combined and secondly, by directly comparing the haplotype frequencies between cases and controls. The log-likelihood ratio tests whether a model where haplotype frequencies in case are different than controls or a model where there are no differences between case and controls fitted the data obtained better(Zhao, Curtis, and Sham 2000). Significance for association was calculated using the test statistic 2*(ln(L case)+ln(Lcontrol)-ln(Lcase/Lcontrol), which has a $\chi 2$ distribution with n-1 degrees of freedom (where n=number of inferred haplotypes). We measured the linkage disequilibrium between SNPs using Cocaphase Software (bioinformatic programmes available and accessed via the Medical Research Council-Rosalind Franklind Centre of Genomic Research website: www.rfcgr.mrc.ac.uk).

The log-likelihood analysis and direct haplotype comparison are complementary methods to detect association in haplotypic datasets. Log-likelihood analysis is used specifically to address the problem of phase uncertainty encountered when direct comparisons are made from haplotype frequencies which are inferred. Direct haplotype comparison assumes that all haplotypes are known without error and therefore can be counted. However, there

remains a degree of uncertainty even in the case of estimated diplotypes. Although, the likelihood based method will test for association, it does not however, provide an estimate of the size of the effect and for this; we used the most likely diplotypes to do so.

5.3 Results

The effect of MDR1 C3435T and G2677T polymorphism on overall disease susceptibility

Both T-allele and TT-genotype of the MDR1 3435 SNP were significantly increased in patients with UC compared with healthy controls (58.2% vs. 52.8%, p=0.02, OR 1.28, CI 1.03-1.58 and 34.6% vs. 26.5%, p=0.04, OR-1.60, CI 1.04-2.44 respectively) (Table 2a). No significant differences in allele or genotype frequencies were seen in patients with CD (53.0% vs. 52.8%, p=0.43 and 26.9% vs. 26.5%, p=0.81 respectively) when compared with controls. A trend towards higher T-allele and genotype frequencies in UC were observed when compared with CD, (58.2% vs. 53.0, p=0.12 and 34.6% vs. 26.9%, p=0.07).

No significant differences in carriage rate (presence of one or two copies of alleles) of 3435T in the 3 groups (UC 81.8%, CD 79.1% and HC 77.8%) were detected. This data suggests that the TT-genotype rather than T-allele carriage play the more significant role in the association with UC. The odds ratio, comparing with the CC-genotype, for the TT-genotype was 1.59 (95% CI 1.04-2.44) and CT-genotype was 1.12 (95% CI 0.76-1.66). Therefore, a stronger significance was also obtained when we compared the homozygosity rate (TT-genotype/non-TT genotype) in UC (p=0.02, OR 1.47, 95% CI 1.06-2.03).

No significant differences were observed for allelic and genotype frequencies for MDR1 G2677T polymorphism on overall disease susceptibility for either UC or CD (Table 2b).

There was no overall association with IBD in the present population for the G2677T SNP (p=0.26) although the G2677 allele frequency showed a trend to be higher in patients with UC compared with controls (54.6% and 51.2% respectively). All genotype frequencies in both cases and controls were consistent with Hardy-Weinberg equilibrium.

In addition to this, I performed a gender matched analysis for both C3435T and G2677T SNP with controls (335 and 268 healthy controls with male frequencies of 48.5% and 43.5% for UC and CD respectively). The association observed with C3435T SNP and UC remained significant with the TT genotype (p=0.05, odds ratio 1.58, 95% CI 1.02-2.45) and T-allele (p=0.03, odds ratio 1.26, 95% CI 1.03-1.06) both significantly increased. No other significant differences were detected in gender-matched analysis (full details available on request).

Linkage disequilibrium between C3435T and G2677T polymorphisms

The C3435T and G2677T SNP are in linkage disequilibrium with each other, in this population. In cases, the D' =0.8, r^2=0.7 and controls, D' =0.9, r^2=0.8 Therefore, we proceeded to perform 2-locus haplotypes association tests with UC and CD.

The effect of 2-locus haplotype (C3435T/G2677T) on disease susceptibility

Using the log-likelihood ratio test as described earlier, I demonstrated an association of 2-locus haplotypes of C3435T/G2677T with UC (p=0.0056, 3 degrees of freedom). On single haplotype analysis, the carriage of 3435T/2677G haplotype conferred an increased risk to UC (odds ratio 1.44, p=0.03, 95% CI 1.03-1.99) (Table 3). In contrast, carriage of

the 3435C/2677T haplotype is associated with a protective effect in UC (odds ratio 0.35, p=0.002, 95% CI 0.17-0.69). Similar trends were observed with the same haplotypes in CD, but these failed to reach significance (3435C/2677T allele, p=0.20 and 3435T/2677G allele, p=0.13).

It is noteworthy that a significantly higher number of patients with UC were homozygotes for both TT 3435 and GG 2677 compared to a low frequency in healthy controls (8 patients possessing 3435TT and GG 2677 genotype vs. 1; p=0.017, OR 8.88 95% CI 1.10-71.45). A similar trend was observed- in patients with CD (5 patients possessing 3435TT and GG 2677 genotype; p=0.08, OR 6.82, 95% CI 0.79-58.77). Combining UC/CD yielded a greater significance, with p-value = 0.013, OR 8.34, 95% CI 1.09-64.07). There were no obvious associations observed in simple and compound heterozygotes of either UC or CD with HC.

As carriers of double homozygote mutants were uncommon (14 individuals in total), we analyzed the phenotypes of these patients. In UC, 6/8 patients had pancolitis of whom 4 required surgery as a consequence of severe disease. Of the 5 patients with CD carrying these 2 mutations, 1 patient had colonic disease (L2), 3 ileo-colonic disease (L3) and 1 ileal disease (L1); 3/5 of these patients required surgery for active disease.

Genotype-phenotype analysis: Univariate analysis

The T-allele and TT-genotype of the MDR1 C3435T showed highly significant association with the phenotype of extensive disease (OR 1.70, 95% CI 1.24-2.29, p=0.009 and OR 2.64, 95% CI 1.34-4.99, p=0.0027 respectively). Both the frequencies of T-allele and TT-genotype of MDR1 C3435T were increased in patients with left-sided colitis and proctitis but these differences were not significant. A significantly higher T-allele frequency was also observed in patients with severe disease (OR 1.39, p=0.04, 95% CI). Although the T-allele and TT-genotype frequencies were even higher in the subgroup of patients who had required surgery for failure of medical treatment for severe UC (T-allele frequency 60.3% and TT-genotype 41.3%), significance was not achieved in this smaller group (Table 4).

Interestingly, for the MDR1 G2677T SNP, trends of association to severe disease and surgery were observed for the GG-genotype and G-allele. In patients with severe disease who required surgery, the GG genotype frequency was 41.2% compared with 27.5% of controls (p=0.11, OR 1.85, 95% CI 0.89-3.85). This trend was not observed in the sub-group of patients with extensive disease.

I did not observe any associations with sub-phenotypic categories of CD (data not shown); specifically colonic CD was not associated with either SNPs (TT genotype frequency, 24.7% vs. 26.5% for controls, p=0.9).

Multivariate analysis

The phenotypes of disease extent, severity and need for surgery were considered in our multivariate model. Multivariate analyses show that the TT genotype of MDR1 3435 remained significant associated with extensive disease (p=0.007). The phenotypes of severe disease and surgery were not significant (p=0.8 and 0.4 respectively). Other models which included age of onset, smoking status, presence of PSC and extraintestinal manifestations revealed no additional associations. No associations were observed with CD.

5.5 Discussion

This study firstly provides replicated confirmation for the association of the MDR1 C3435T SNP with UC following the initial case-control study reported by Schwab et al. In addition, I have made novel observations with respect to genotype-phenotype correlations, notably the strong association of the C3435T SNP with extensive UC. Finally, the haplotypic analyses involving C3435T and G2677T SNPs provide further new insights into the complexities of the contribution of the MDR1 gene – both protective and susceptible haplotypes were identified.

Indeed in view of the strength of the phenotypic associations identified in the present study, it is of interest to reconsider whether the positive association with C3435T SNP seen in Schwab's study (43% of patients had extensive UC in that cohort) may have been driven by this phenotype. A significant association with extensive UC was not detected in

that study but that may have been due to lack of statistical power in a smaller sub-group (n=63). This highlights the important point of phenotypic heterogeneity when considering future association studies in this and other candidate genes in IBD.

The haplotypic data provide further insight into the contribution of the MDR1 gene in determining susceptibility, and disease phenotype. By combining the 2 SNPs, the haplotype 3435T/G2677 was shown to confer an increased susceptibility to UC (p=0.03, OR 1.44, 95% CI 1.03-1.99) whereas haplotype C3435/2677T appeared to protect against the development of UC (p=0.002, OR 0.35, 95% CI 0.17-0.69). The effect was most pronounced in patients who were homozygotes for both MDR1 3435 TT and 2677 GG genotype but we note that the overall number of this group was very low (2.4%). The likelihood ratio for the haplotypic distribution in UC was also significantly different when compared with controls (p=0.0056). Thus, it appears that these variants can alter the risk of developing UC in a bi-directional fashion. It is particularly interesting that the at-risk haplotype contains allelic variants associated with reduced P-glycoprotein expression in vivo, whereas the alleles on the protective haplotype have been associated with increased expression.

In the recent years, the case for the involvement of the MDR1 gene and P-glycoprotein 170 in determining susceptibility in IBD has become increasingly persuasive. It is clear however that the role of the MDR1 gene/P-glycoprotein 170 in inflammation is likely to be more complex than originally thought. Most pertinently, there appears to be cell, tissue and even regional organ specific differences in the regulation of both the function and

expression of P-glycoprotein 170(Yacyshyn, Maksymowych, and Bowen-Yacyshyn 1999; Lin et al. 1999; Zhao et al. 1993; Murakami et al. 2002; Moodie et al. 2003). Increasingly, *in vitro* and *ex vivo* studies, including data from our unit involving the HLA-B27 transgenic mice suggest that P-glycoprotein expression is in fact reduced in the presence of colonic inflammation(Moodie et al. 2004; Mizoguchi et al. 2003; Iizasa et al. 2003). This together with the findings by Langmann et al., demonstrating down-regulation of MDR1 (with other detoxification genes) in colonic tissue of patients with UC, puts forward a compelling argument for an influential role of P-glycoprotein in determining susceptibility to UC(Langmann et al. 2004). We hypothesize that low levels of P-glycoprotein 170 expression in the GI colonic epithelium increases susceptibility and high levels are protective.

Based on our data, the C3435T and not G2677T SNP is primarily associated with UC. This leads on to the question whether C3435T is the functional variant or in linkage with another variant. Given that we did not show an association with G2677T alone and that 2-locus haplotypes were not superior in determining risk, we clearly cannot ascribe the significant association seen with C3435T to be secondary to linkage with G2677T/A. The functional effect of the C3435T, a synonymous SNP that does not involve amino-acid change nevertheless remains controversial. It remains possible that this silent SNP can affect P-glycoprotein 170 activity/expression through for example, effects on mRNA stability or codon preference. However, it is pertinent that the correlation between C3435T SNP and P-glycoprotein 170 activity/expression does not appear consistent across ethnic groups. Studies in Caucasian populations showing an association with

MDR1 3435 TT-genotype and lowered P-glycoprotein 170 activity/expression, but the reverse is true for studies involving Japanese populations (Nakamura et al. 2002; Drescher et al. 2002; Sakaeda et al. 2001). Therefore, we propose that the argument that this SNP lies within tight LD with another unidentified causal variant remains the most plausible explanation.

Does MDR1 have a role as a pharmacogenetic marker? Farrell and colleagues, suggested that high P-glycoprotein 170 expression was associated with failure of medical treatment in IBD(Farrell et al. 2000). The study did not investigate the genetic contribution of the MDR1 gene. The stratification to severe disease and need for surgery in our study, was originally driven by the hypothesis that the CC-genotype (in which some studies have shown to be associated with high expression) can predict corticosteroid resistance (severe disease) and therefore surgery. This in fact was not shown in our study with the trend being completely reversed (MDR1 3435-TT genotype higher in both patients with severe disease and requiring surgery). Recent data from the Oxford dataset in abstract form even suggest that the TT-genotype of the MDR1 3435 SNP may in fact be useful to predict surgery in UC, in conjunction with other genetic markers(McGovern et al. 2004).

Are allelic variants of the MDR1 gene implicated in Crohn's disease? Overall, I did not detect any associations with CD and the sub-phenotypes according to Vienna Classification of age of onset, disease location and behaviour (data not shown). In particular, no association was seen when I specifically considered only Crohn's colitis with either of the 2 SNPs. There were however, trends of associations with 2-locus

haplotype (haplotypes 3435T/2677T, OR-0.85, p=0.18; C3435/2677T, OR-1.39, p=0.19; and 3435T/G2677, OR-1.29, p=0.16). This CD population is smaller, and given the heterogeneity involved in the presentation of CD, there may be statistical limitations in conclusively confirming or refuting the hypothesis of association with CD.

In conclusion, this study provides robust evidence to support a role of the MDR1 gene in the pathogenesis of UC. Germline MDR1 variation determines both disease susceptibility, and course in the Scottish population. The data point to the presence of more than one functional variant or a more haplotype-specific effect, and underline the need for parallel functional studies, and haplotype analyses (Soranzo et al. 2004).

Table 1a: Demographics and clinical characteristics of UC study population (n=335) *- Severe UC defined as patients who had developed severe acute attack of UC satisfying the Truelove and Witts criteria.

Characteristics	UC
Sex (M/F)	184/151
Age of onset (years)	35.0 (25.3-50.3)
Age of onset <16 years	10 (3%)
Current smoker Ex-smoker Never smoked	28 (8.5%) 135 (40.4%) 172 (51.4%)
Ethnicity Scottish Caucasian Others	 99% (1 Jew, 1 Japanese and 2 Asians)
Disease extent Extensive disease (>Splenic flexure) Left-sided colitis Proctitis Severe disease* Surgery for severe disease	 118 (35.2%) 149 (44.5%) 68 (20.3%) 113 (33.7%) 63 (18.8%)
Extra-intestinal manifestations Primary Sclerosing Cholangitis	31 (9.4%) 6 (1.8%)
Drug therapy Azathioprine	 48 (14.2%)

Table 1b: Demographics and clinical characteristics of patients with CD. Disease location, behaviour and age of onset were defined according to the Vienna Classification.

Characteristics	Crohn's disease
Sex (M/F)	117/151
Smoking Current Ex-smoker Never	 68 (25.4%) 77 (28.7%) 123 (45.9%)
Age of onset, years Median (+IQR)	26.6 (19.9-37.0)
Age of onset (<40 years, A1)	203 (75.7%)
Ethnicity Scottish Caucasian Others	 99% (1 Jewish, 2 Asians)
Drug therapy Infliximab Azathioprine	 45 (16.8%) 106 (39.5%)
Disease Location (n=240) Ileal (L1) Colonic (L2) Ileo-colonic (L3) Upper GI (L4)	 83 (30.9%) 118 (39.9%) 57 (21.3%) 21 (7.9%)
Disease behaviour *(at diagnosis)* Inflammatory (B1) Stricturing (B2) Penetrating (B3) *(at latest follow-up)* Inflammatory (B1) Stricturing (B2) Penetrating (B3)	 196 (73.1%) 21 (7.8%) 51 (19.0%) 95 (35.4%) 43 (16.1%) 130 (48.4%)
Surgery	122 (45.8%)
Extra-intestinal manifestations	59 (22.1%)

Table 2a: Genotype and allelic frequencies of MDR1 C3435T polymorphism in UC and CD, compared with controls. UC vs. CD: TT-genotype, p=0.12, OR 1.48, 95% CI 0.93-2.36 and T-allele, p=0.07, OR 1.24, 95% CI 0.98-1.24.

	CC (%)	**CT (%)**	**TT (%)**	**C (%)**	**T (%)**	**TT vs. CC Odds ratio (95% CI)**	**T vs. C Odds ratio (95% CI)**
UC (n= 335)	61 (18.2)	158 (47.2)	116 (34.6)	280 (41.8)	390 (58.2)	0.04 1.60 (1.04-2.44)	0.02 1.28 (1.03-1.58)
CD (n=268)	56 (20.9)	140 (52.2)	72 (26.9)	252 (47.0)	284 (53.0)	0.81 1.08 (0.68-1.69)	0.43 1.03 (0.83-1.29)
Healthy controls (n=370)	82 (22.2)	190 (51.3)	98 (26.5)	354 (47.8)	386 (52.8)		

Table 2b: Genotype and allele frequencies of MDR1 G2677T polymorphism in UC and CD, compared with controls. UC vs. CD: GG-genotype, p=0.48, OR 1.19, 95% CI 0.75-1.89 and G-allele, p=0.19, OR 1.15, 95% CI 0.93-1.89.

	GG (%)	**GT (%)**	**TT (%)**	**G (%)**	**T (%)**	**GG vs. TT Odds ratio (95% CI)**	**G vs. T Odds ratio (95% CI)**
UC (n= 335)	95 (28.3)	176 (52.5)	64 (19.1)	366 (54.6)	304 (45.4)	0.16 1.37 (0.89-2.09)	0.19 1.15 (0.93-1.42)
CD (n=268)	75 (27.9)	133 (47.8)	60 (22.4)	283 (52.8)	253 (47.2)	0.57 1.15 (0.74-1.79)	0.57 1.07 (0.86-1.34)
Healthy controls (n= 370)	102 (27.6)	174 (47.0)	94 (25.4)	378 (51.2)	362 (49.8)		

Table 3: 2-locus haplotypes of C3435T/G2677T SNPs with UC/CD. Contingency tests were used to test the association of the inferred haplotypes in the groups of UC and CD respectively as compared with the control group (total number of a particular haplotype/total number of remaining haplotypes compared between case and controls with p-values and 95% confidence intervals given).

Haplotype	UC (%)	HC (%)	P-value OR (95% CI)	CD (%)	HC (%)	P-value OR (95% CI)
3435T/2677T	291/379 43.4	332/408 44.9	0.59 0.94 (0.76-1.16)	219/317 40.8	332/408 44.9	0.16 0.85 (0.67-1.06)
C3435/G2677	276/394 41.2	300/440 40.6	0.82 1.03 (0.83-1.27)	221/315 41.2	300/440 40.6	0.82 1.03 (0.82-1.29)
3435T/G2677	91/579 13.5	73/667 9.8	**0.03** 1.44 (1.03-1.99)	65/471 12.1	73/667 9.8	0.20 1.26 (0.88-1.79)
C3435/2677T	11/659 1.7	34/706 4.6	**0.002** 0.35 (0.17-0.69)	35/501 5.9	34/706 4.6	0.13 1.45 (0.89-2.36)

Table 4: Genotype-phenotype analysis for C3435T and G2677T SNP in UC

C3435T					
Genotypes	CC	CT	TT	TT-genotype P-value Odds ratio	T-allele frequency P-value Odds ratio
Extensive UC	17 (14.4%)	51 (43.2%)	50 (42.4%)	0.003 2.64 (1.34-4.99)	0.009 1.70 (1.24-2.29)
Left sided disease	30 (20.1%)	74 (49.7%)	45 (30.2%)	0.49 1.25 0.73-2.17	0.41 1.12 0.86-1.47
Proctitis	14 (20.6%)	37 (54.4%)	21 (30.9%)	0.58 1.25 0.60-2.62	0.57 1.11 0.79-1.59
Severe disease	23 (20.3%)	44 (38.9%)	46 (40.7%)	0.09 1.67 (0.94-2.99)	0.04 1.39 (1.02-1.88)
Surgery for severe disease	13 (20.6%)	24 (38.1%)	26 (41.3%)	0.21 1.67 (0.81-3.46)	0.18 1.31 (0.89-1.91)
G2677T					
Genotypes	GG	GT	TT	GG-genotype P-value Odds ratio	G-allele frequency P-value Odds ratio
Extensive UC	32 (27.1%)	60 (50.9%)	26 (22.0%)	0.76 1.13 (0.63-2.04)	0.71 1.06 (0.79-1.42)
Left sided disease	44 (29.5%)	80 (53.7%)	25 (16.8%)	0.12 1.62 0.92-2.85	0.13 1.24 0.94-1.62
Proctitis	19 (27.9%)	36 (52.9%)	13 (19.1%)	0.57 1.35 0.63-2.88	0.51 1.14 0.79-1.65
Severe disease	35 (31.0%)	53 (47.8%)	20 (21.2%)	0.17 1.61 (0.87-2.99)	0.14 1.27 (0.93-1.72)
Surgery for severe disease	26 (41.2%)	24 (38.1%)	13 (20.6%)	0.11 1.84 (0.89-3.79)	0.06 1.46 (0.99-2.14)

Chapter 6

The ABCB1/MDR1 gene determines susceptibility and phenotype in ulcerative colitis: definition of critical variants using a gene-wide haplotype tagging approach

Abstract

Background: Several lines of evidence suggest a role for the multidrug resistance gene (*ABCB1/MDR1*) in the pathogenesis of inflammatory bowel disease (IBD).

Aims: To investigate the overall contribution of MDR1 gene to susceptibility of UC and CD using a gene-wide haplotype tagging approach.

Methods: Six haplotype tagging SNPs (tSNPs) representing the haplotypic variations of the ABCB1/MDR1 gene were identified initially following the characterization of the haplotype structure of this gene in 24 CEPH Caucasian trios. Genotyping was performed in 249 ulcerative colitis (UC) and 179 Crohn's disease (CD) patients and 260 healthy controls (HC).

Results: Using log-likelihood analysis, a highly significant association between the common haplotypes and UC ($p=4.22x10^{-7}$) was observed but not with CD (p=0.22). This significant association was critically dependent on one tSNP, intronic variant rs3789243. All haplotypes with this variant retained a highly significant association ($p= 3.2 \times 10^{-7}$ - 3.6×10^{-12}) whereas significance was lost when rs3789243 was dropped in systematic haplotypic analysis. The effect of this tSNP was independent of C3435T SNP (previously shown to be associated with UC). The association with UC was also shown to be strongest with the phenotype of extensive disease ($p=1.7 \times 10^{-7}$).

Conclusion: This 'candidate gene' approach provides the strongest yet evidence to support the contribution of the ABCB1/MDR1 gene in determining risk to UC but not CD in our population.

6.1 Introduction

As demonstrated in chapter 5, the candidate polymorphism C3435T of the MDR1 gene is associated with UC and extensive disease in the Scottish dataset. Although this SNP has been shown to be associated with expression of P-glycoprotein 170, the mechanisms whereby this variant (and the G2677T/A SNP) affect gene transcription has yet to be clarified. There are several reasons to suspect that the effect seen can be ascribed to linkage disequilibrium with an unknown causal or set of variants. The C3435T SNP is a silent wobble mutation. Site-specific mutagenesis experiments have demonstrated that the C3435T and G2677T/A substitution have no effect on P-glycoprotein function in vivo (Morita, Yasumori, and Nakayama 2003). Haplotypes derived from these two SNPs showed a stronger association with P-glycoprotein activity (inferred from digoxin uptake)(Johne et al. 2002a). Furthermore, the correlation between C3435T SNP and P-glycoprotein activity/expression does not appear consistent across ethnic groups. Studies in Caucasian populations have shown a general trend of association between the MDR1 3435 TT-genotype and lowered P-glycoprotein activity/expression, whereas this association appears generally reversed in the Japanese(Nakamura et al. 2002; Drescher et al. 2002; Sakaeda et al. 2001).

These candidate polymorphisms have now been studied extensively in several other datasets since our initial study. Presently, a total of nine studies have examined the effect of these candidate SNPs (C3435T and G2677T) either singly or in combination in determining susceptibility to inflammatory bowel disease (Table 1)(Schwab et al. 2003c; Brant et al. 2003; Croucher et al. 2003; Glas et al. 2004; Potocnik et al. 2004; Ho et al.

2005). The overall results have been inconsistent: 3 studies have reported no associations with either CD or UC, a further 4 studies demonstrated associations with UC and a further 2 studies demonstrated associations with CD. Several factors are pertinent when considering the apparently inconsistent results from these studies. The two most important points which may confound the overall picture are phenotypic (given previous association with extensive disease) and genetic heterogeneity.

To obtain a more robust assessment of the contribution of the ABCB1/MDR1 gene to disease susceptibility, we utilized an approach where highly informative 'tagging' SNPs (tSNPs) which represent the haplotypic variations of this gene are used to test for association. This involved a three-step approach where firstly the ABCB1/MDR1 haplotype structure was initially characterized by re-sequencing this gene in 24 CEPH Caucasian trios. Having established the pattern of LD, we then identify a set of SNPs which represent or 'tag' the common variations of the region, in this case, the gene. We then genotyped the tSNPs in the study population and employ likelihood calculations to determine whether there is a difference between case and control populations. In this study, we have identified 6 tSNPs by using a selection strategy which is 'block' free initially described by Weale et al (Weale et al. 2003). This is therefore a 'gene'-wide study and also implicitly a candidate gene rather than a candidate polymorphism study.

6.2 Material and Methods

Patients and controls

A total of 249 patients with UC and 170 patients with CD were studied together with 260 healthy controls. Table 2 summarizes the clinical characteristics of studied patients. The median ages of diagnosis for UC and CD were 35.8 years (IQR 26.4-50.2) and 27.3 years (IQR 21.0-42.7) respectively. The ethnicity of our study population in both cases and controls were Scottish-Caucasians. There were more males in the UC cohort (54.5%) and fewer in CD (44.5%). These differences were not significant when compared with controls In the UC group, 42% and 29% of patients had extensive and severe disease respectively.

Re-sequencing of the ABCB1/MDR1 gene and the selection of tSNPs

The re-sequencing of 12 amplicons distributed along the length of the ABCB1/MDR1 gene and corresponding to a total of 4.1 kb identified 17 SNP loci in 24 Caucasian CEPH trios (Soranzo et al. 2004)(Table 3). Three loci had a low minor allele frequency (<6%) and were therefore excluded from further analysis as these low frequency variants were unlikely to be responsible for the previously documented observed association between UC and the common C3435T SNP(Ho et al. 2005; Schwab et al. 2003a). Using 14 SNPs with high minor allele frequency, 2114 haplotypes were obtained where only haplotypes with frequencies >1%, equating to 39 common haplotypes were used for further analysis (corresponding to 95% of the total haplotypic variation of this gene). A set of 6 tSNPs were identified that provide a coefficient of determination of at least 0.80 in predicting all the known SNPs (Table 4) (Weale et al. 2003; Goldstein et al. 2003).

The criteria for the choice of tSNPs were i) average r^2 weighted by allele frequency between the tSNPs and all SNPs were ≥ 0.80 and ii) minimum r^2 between the tSNPs and each individual SNPs in the gene was 0.80. All genotype data was entered into the TagIT program (criterion 5 in TAGIT 3.00 at www.genome.duke.edu/research/centers/pg2) with a minimum haplotype r2 of 0.80 to select the tSNPs. Haplotype r^2 is the coefficient of determination from a regression model of an allelic or haplotype state in question against haplotypes determined by tSNPs' set. This method does not rely on heuristically defined haplotype block structure. All genotyping were done using TaqMan technology (sequence reactions available on request).

Genotyping

Genotyping of the subjects in the study was performed using TaqMan technology ($tSNP_{1, 2, 3, 4}$) (ABI, San Diego, CA) or resequencing of PCR products (rs3789243, $tSNP_{5, 6}$) using ABI 3700 Big Dye Terminators. Sequences for tags 11 and 12 were scored twice independently using the Sequencher software (Gene Codes Corporation, Ann Arbour, Michigan, USA). The sequence reactions were described in Chapter 2.

Statistical analysis

The statistical methods to compare allelic and genotypic frequencies were described in the previous chapter. Haplotype frequencies of the ABCB1/MDR1 tSNPs were inferred using the expectation-maximization (EM) algorithm implemented in SNPHAP program(Zhao, Curtis, and Sham 2000). Haplotypic associations with disease were assessed in two ways: 1) A log-likelihood ratio analysis was implemented in the EH and

PM programs (bioinformatic programmes available and accessed via the Medical Research Council-Rosalind Franklind Centre of Genomic Research website: www.rfcgr.mrc.ac.uk)(Zhao, Curtis, and Sham 2000; Qin, Niu, and Liu 2002). This involves the calculation of the log-likelihoods of estimated frequencies for each cases, controls and case and controls combined. Significance for association is calculated using the test statistic 2*(ln(L case)+ln(Lcontrol)-ln(Lcase/Lcontrol), which has a χ^2 distribution with n-1 degrees of freedom (where n=number of inferred haplotypes). 2) Contigency tests were also used to test the association for all haplotypes showing a frequency difference between cases and controls. We measured the linkage disequilibrium between SNPs using Cocaphase Software (also accessed via the Medical Research Council-Rosalind Franklind Centre of Genomic Research website: www.rfcgr.mrc.ac.uk).

6.3 Results

Based on the haplotypic information from the initial re-sequencing of 24 CEPH trios, we have selected a set of 6 tSNPs with good performance (with a coefficient of determination of at least 0.80 in predicting all the known SNPs) (Table 4) (Weale et al. 2003; Goldstein et al. 2003); and genotyped these tSNPs in a panel of 249 and 179 individuals with UC and CD respectively and 260 healthy controls in a Scottish-Caucasian population. The genotypic frequencies of the 6 selected tSNPs are shown in Table 5. The G-allele of rs3789243, $tSNP_1$ (p=0.03, OR 1.31, 95% CI 1.03-1.68) and GG-genotype (p=0.04, OR 1.76, 95% CI 1.06-2.92) were significantly higher in patients

with UC compared with controls. No other significant differences were observed in UC or CD.

Using the log-likelihood ratio analysis which tests the association between the inferred haplotypes using disease/control partition (using EH+ which makes a likelihood assessment that includes statistical uncertainty in phase estimation), I detected a strong association of haplotypes with UC, with a highly significant p-value of 4.22×10^{-7} (35 degrees of freedom). In contrast, no association was seen with CD (p=0.22). The log-likelihood analysis was most significant in the phenotype of extensive UC (colitis extending beyond the splenic flexure) ($p=1.7 \times 10^{-7}$), and less in recto-sigmoid disease (0.0089) (Table 6). No association was seen within the sub-phenotypes of disease location and behaviour in CD (data not shown).

I further analyzed all combinations of haplotypes to (i) determine which tSNP/s is/are responsible for the observed association and (ii), whether the effect observed is haplotype specific. I therefore performed a log-likelihood analysis on all possible sets of 2-, 3-, 4- and 5-locus haplotypes (a total of 56 different sets of haplotypes). Only haplotypes containing rs3789243, $tSNP_1$ were found to retain a significant association with UC (Table 7). It is particularly noteworthy that in the 26 haplotypes without rs3789243, $tSNP_1$, none of these approached significance.

Eleven haplotypes (from a total of thirty) containing rs3789243, $tSNP_1$ but without rs1045642, $tSNP_4$ (C3435T), demonstrated highly significant association. Conversely,

there was no association seen in the total of 15 haplotypes which contained rs1045642, $tSNP_4$ (C3435T), but not rs3789243, $tSNP_1$. This imply that rs1045642, $tSNP_4$ (C3435T) is not responsible for the overall association seen using the 6-locus haplotype. We have focussed on rs1045642, $tSNP_4$ (C3435T) due to the controversies surrounding the contribution of this SNP to disease susceptibility as highlighted in Table 1.

Having found an overall association with UC, I systematically examined the direct haplotypic frequencies (as inferred using the PL-EM program). For each of the 2, 3, 4 and 5-locus haplotypes, we have selected and compared their inferred respective haplotypic frequencies between the groups of UC and controls. These specific haplotypes which were most significantly associated with UC are shown in Table 8. It is pertinent that these haplotypes all contained the G-allele of rs3789243, $tSNP_1$ .again confirming the critical importance of this tSNP. Similarly, the strongest effect was determined by 2-locus haplotype composed of $tSNP_1$ and $tSNP_5$, with an odds ratio of 3.27 ($p < 0.00001$, 95% CI 2.26-4.72).

6.4 Discussion

This 'gene-wide' approach provides the most robust evidence yet to support the genetic contribution of the ABCB1/MDR1 gene to the susceptibility of UC. I observed a highly significant association (log-likelihood ratio test $p = 4.22 \times 10^{-7}$) with UC and not CD in our population. As this study accounted for the haplotypic variations across the gene, the results are much stronger than the previously published studies utilizing candidate polymorphisms, C3435T and G2677T/A(Ho et al. 2005; Schwab et al. 2003a; Croucher

et al. 2003; Brant et al. 2003; Glas et al. 2004; Potocnik et al. 2004). In addition, we have again shown that the contribution of germ-line variations of the ABCB1/MDR1 gene is primarily most significant in the phenotype of extensive colitis. There remains a degree of controversy of whether the ABCB1/MDR1 gene contributes only to disease susceptibility in UC and not CD. Previous genetic studies have presented a more inconsistent picture (Table 1). The negative result which we have found from our haplotypic approach sets a statistical limitation to the importance of common variants in this gene and their contribution to CD-susceptibility in our population.

Confirming the initial impression of the complex contribution of the ABCB1/MDR1 gene, this data shows that the highly significant association is critically dependent on an upstream intronic variant rs3789243, $tSNP_1$ which is also independent from the effect of the often cited rs1045642, $tSNP_4$ (C3435T). Although it is not possible to conclusively disprove the case for the rs1045642, $tSNP_4$ (C3435T) as the 'causal' variant for previously observed association, this finding strongly suggest this is not likely to be the case. This lends considerable support to the current thinking that C3435T and G2677T/A (in exons 26 and 21 respectively) are not the causal variants but lie in linkage disequilibrium with it/them. The replication at the haplotype level based on the tSNPs identified by our study, in other European Caucasian populations is now crucial and will be more powerful than further candidate SNP analysis in this field in IBD, as already more extensive variation has been studied and tagged in this essentially gene-based study(Neale and Sham 2004).

The 'block' free haplotype tagging approach utilized by this study, which circumvents the heuristically defined block boundaries, originally described by Weale and Goldstein(Weale et al. 2003; Goldstein et al. 2003) have been shown recently following extensive empirical evaluations to be efficient and effective method to represent both known and unknown common variations(Ahmadi et al. 2005). In the case of this study, I have successfully used this method in an area of which controversy still exist e.g. the genetic contribution of the ABCB1/MDR1 gene to susceptibility of IBD. In this current approach, I have not determined the extent of LD outwith the ABCB1/MDR1 gene. Therefore, it is conceivable that this set of tSNPs can also pick up long range LD signals from neighboring regions in contrast to the more conventional concept of tagging major haplotypes in blocks of high LD(Kamatani et al. 2004). Nevertheless, with the data from the mdr-1a knock-out models and other expression studies both in human and animals which strongly implicate the role of P-glycoprotein 170, the product of the ABCB1/MDR1 gene in the aetiopathogenesis of IBD, it is less likely that the strong signal that we have obtained are derived from outwith the gene (Panwala, Jones, and Viney 1998; Langmann et al. 2004; Maggio-Price et al. 2002; Maggio-Price et al. 2005) (Iizasa et al. 2003; Mizoguchi et al. 2003).

Future work should be directed towards deep re-sequencing the associated interval surrounding rs3789243, $tSNP_1$ in a cohort of patients with extensive UC, of which we have demonstrated to be the sub-phenotype most implicated by this gene. Although I have further fine localized the region of interest in this gene, the ease or difficulty in pin-pointing the 'causal' variant/s remains unpredictable. It is possible that the extent of

linkage disequilibrium could be so high that the 'causal' variant could lie anywhere in the block on the associated chromosome. This difficulty has been highlighted in the recent studies focusing on the IBD5 locus, cytokine gene cluster in chromosome 5q31-33, a 250 kB haplotype of high LD which conferred susceptibility to CD (Peltekova et al. 2004; Newman et al. 2005). It seems increasingly certain that once haplotypic replication has been attained in the case of ABCB1/MDR1 gene, functional studies examining the effect of the list of putative casual variants derived from further fine localization will be the direction to take.

It is pertinent that the implications of these findings may extend beyond the field of inflammatory bowel disease. As suggested earlier, there has been much interest surrounding the issue of the identification of the 'causal' variant/s underlying reported genetic associations of the candidate SNP rs1045642, $tSNP_4$ (C3435T), with other clinical conditions such as drug-resistant epilepsy(Siddiqui et al. 2003; Soranzo et al. 2004), immune recovery after initiation of anti-retroviral therapy in HIV(Fellay et al. 2002), increased risk to renal cell carcinoma(Siegsmund et al. 2002) and other drug responses in the field of pharmacogenetics. It would be of great interest to examine the contribution of these tSNPs in these other conditions. The economy and applicability afforded by this approach is now well-demonstrated. If variations of the ABCB1/MDR1 gene are indeed implicated in these conditions, similar degrees of associations can be anticipated.

By using a 'gene-wide' haplotype approach which is increasingly accepted as the model for future genetic association studies(Neale and Sham 2004), we have now provided very

strong evidence to implicate the genetic involvement of the ABCB1/MDR1 gene in susceptibility to UC but not CD in our population. This data also crucially enable further targeted fine localization in the search for the critical 'causal' variant for the observed association in our population.

Figure 1: The extent of linkage disequilibrium of ABCB1/MDR1 gene.

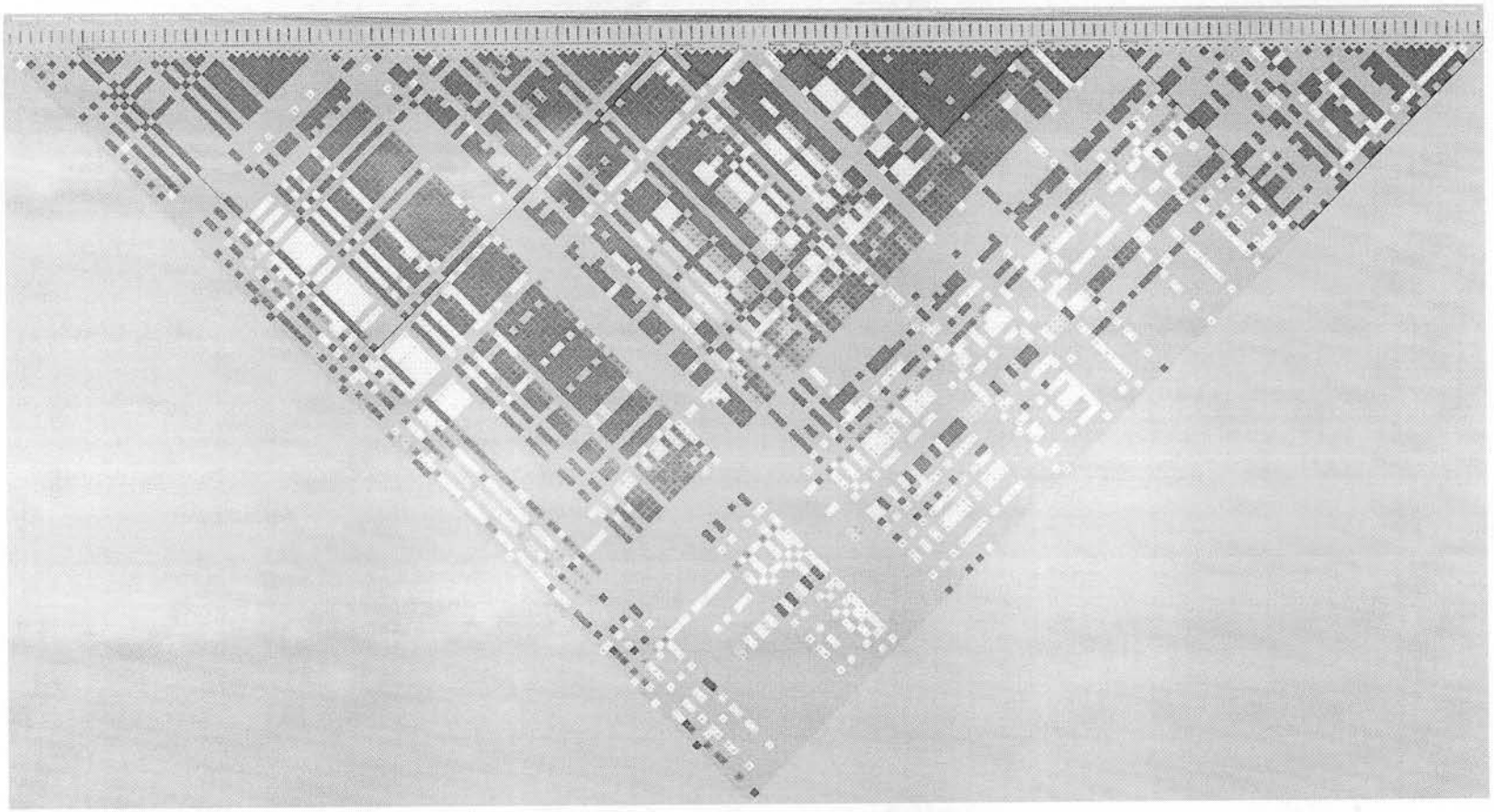


Table 1: Summary of studies investigating the contribution of allelic variants of the ABCB1/MDR1 gene to susceptibility of inflammatory bowel disease. TDT- transmission, SNP-single nucleotide polymorphism, PDT- pedigree disequilibrium test.

Author	Design	Ethnicity	Findings
Schwab et al. 2003	Age and sex matched case-control study of C3435T SNP only	German	T-allele and TT-genotype of the C3435T SNP associated with UC (p=0.049), not CD
Croucher et al. 2004	Family based TDT and case-control study of C3435T SNP only	German and British families	No association with C3435T with either UC or CD
Glas et al. 2004	Case-control of C3435T SNP	Caucasian	No association with C3435T with either UC or CD
Brant et al. 2004	Case-control and family based TDT of C3435T, G2677T SNP and C1236T SNP	Mixed North American Caucasian and Jewish families	Association with G-allele of G2677T SNP with IBD (p=0.002) in case-control analysis and PDT (p=0.0002-0.003). Undertransmission of 2677T and A variants.
Potocnik et al. 2004	Case-control and haplotypic analysis of C3435T, G2677T SNP and C1236T SNP	Slovenian	Haplotypic association with refractory CD and UC
Ho et al. 2005	Case control study of C3435T and G2677T SNP With sex-matched stratification	Scottish	T-allele and TT-genotype of MDR1 3435 with UC (p=0.02 and 0.04 respectively). Strong association with extensive UC (p=0.003). No association with G2677T. Protective and risk conferring haplotypes derived from 2 studied SNPs.
Palmieri et al. 2005	Case control study of 478 CD, 468 UC and 450 HC (C3435T and G2677T/A SNPs)	Italian	No associations with CD, UC or response to medical therapy
Urcelay et al. 2006	Case-control study of 321 CD, 330 UC and 352 ethnically matched controls	White Spanish	The MDR1 C3435T allele and 2677T/C3435 haplotype were significantly associated with CD (p = 0.007)
Onnie et al. 2006	Case-control study of 828 CD, 580 UC and 285 healthy controls	British	The 2677T allele was significantly associated with UC (p = 0.034). The 2677 TT genotype was significantly associated with severe UC and steroid usage in UC

Table 2: Demographics of patients studied. Extensive disease was classified as disease > splenic flexure, severe disease classified as patients who had developed severe attack of UC which satisfied the Truelove and Witts criteria. For CD, disease location and behaviour were classified according to the Vienna Classification(Gasche et al. 2000). Disease behaviour was taken from time of diagnosis. Both groups comprised of Scottish Caucasians.

Clinical features	**UC (n=249)**	**CD (n=179)**
Sex (Females)	45.5%	55.5%
Age of diagnosis (Median +IQR)	35.8 26.4-50.2	28.9 21.0-42.7
Smoking Current Never Ex-smoker	 9.1% 49.3% 41.6%	 28.4% 45.9% 25.7%
Disease extent/location	Extensive disease– 41.7%	Ileal (L1) – 39.1% Colonic (L2) – 30.7% Ileocolonic (L3) – 20.1% Upper GI (L4) – 6.1%
Disease behaviour	Severe disease – 29.7%	Inflammatory (B1)-72.9% Stricturing (B2) -8.0% Penetrating (B3) -19.1%
Need for surgery	20.0%	45.8%

Table 3: List of SNPs used for linkage analysis of MDR1/ABCB1 in 24 CEPH trios. Position – SNP position within the reference contig (Genbank acc. No. NT_007933). *P-values for test of fit to Hardy-Weinberg equilibrium.

No.	Position	dbSNP ID	Location	alleles	F("1")	F("2")	F(1/1)	F(1/2)	F(2/2)	P(chi2)*
1	12455059	rs3789243	Intron 3	A/G	0.4886	0.5114	0.25	0.4773	0.2727	0.7655
2	12438013	rs1202180	Intron 4	A/G	0.6889	0.3111	0.4889	0.4	0.1111	0.654
3	12430135	rs1202168	Intron 6_1	C/T	0.5357	0.4643	0.2143	0.6429	0.1429	0.0582
4	12430023	rs1202169	Intron 6_3	A/G	0.5357	0.4643	0.2143	0.6429	0.1429	0.0582
5	12413774	rs1128503	Exon 12_1	C/T	0.5435	0.4565	0.2391	0.6087	0.1522	0.1242
6	12408239	rs2235046	Intron 18	C/T	0.4674	0.5326	0.1522	0.6304	0.2174	0.0709
7	12394791	rs2032582	Exon 21	G/T,A	0.4722	0.4931	0.2083	0.5278	0.1944	0.3699
8	12372818	rs1045642	Exon 26 C3435T	C/T	0.3958	0.6042	0.1042	0.5833	0.3125	0.1281
9	12371191	rs1882478	Intron 26_2	C/T	0.75	0.25	0.5455	0.4091	0.0455	0.5465
10	12371188	-	Intron 26_1	C/T	0.7727	0.2273	0.5909	0.3636	0.0455	0.8149
11	12368166	rs1186746	Intron 27_9	C/T	0.1915	0.8085	0.0426	0.2979	0.6596	0.7944
12	12368120	rs1186745	Intron 27_5	A/C	0.117	0.883	0	0.234	0.766	0.3636
13	12356195	rs2178658	11 Kb downstream'_2	G/T	0.7021	0.2979	0.4468	0.5106	0.0426	0.1301
14	12356135	-	11 Kb downstream_1	A/G	0.1979	0.8021	0.0417	0.3125	0.6458	0.9133

Table 4: The haplotype r2 of the set of selected 6 tSNPs against each of the SNPs identified as shown in Table 2.

SNP No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
r^2	1	0.92	1	1	1	1	0.94	1	0.80	0.80	1	1	1	1

Table 5: Tagging SNPs and genotype/allelic frequencies in study population.

dbSNP ID	Location	Allele (1/2)	Haplotype tags	UC (N=249)	CD (N=179)	HC (N=260)	1 vs. 2 P-value odds ratio 95% CI	1/1 vs. 2/2 P-value odds ratio 95% CI
rs3789243	Intron 3	G/A						
			AA	18.5%	22.9%	24.2%	0.03*	0.04*
			AG	50.6%	51.9%	52.7%	1.31	1.76
			GG	30.9%	25.1%	23.1%	1.03-1.68	1.06-2.92
rs1128503	Exon 12_1	C/T						
			CC	28.1%	25.1%	29.2%	0.75	0.79
			CT	51.4%	54.7%	51.5%	1.04	1.09
			TT	20.5%	20.2%	19.6%	0.81-1.33	0.65-1.80
rs2235046	Intron 16	C/T						
			CC	26.9%	24.6%	28.5%	0.57	0.62
			CT	49.8%	54.7%	50.4%	1.08	1.16
			TT	23.3%	20.7%	21.1%	0.84-1.38	0.71-1.91
rs1045642	Exon 26 C3435T	T/C						
			CC	18.5%	16.8%	21.1%	0.07	0.12
			CT	46.9%	58.1 %	53.1%	1.26	1.54
			TT	34.6%	25.1%	25.8%	0.98-1.62	0.92-2.54
rs1186746	Intron 27_9	T/C						
			CC	2.4%	3.3%	3.5%	0.67	0.60
			CT	26.5%	26.3%	26.5%	1.08	1.46
			TT	71.1%	70.4%	70.0%	0.77-1.51	0.51-4.81
rs1186745	Intron 27_5	C/A						
			AA	6.4%	5.0%	7.7%	0.24	0.47
			AC	39.8%	38.5%	44.2%	1.19	1.34
			CC	53.8%	56.4%	48.1%	0.90-1.56	0.66-2.70

Table 6: Log-likelihood significance between the common haplotypes of ABCB1/MDR1 haplotypes and UC/CD. Extensive disease – colitis extending beyond the splenic flexure; and left sided disease-colitis limited to the splenic flexure. No significant association demonstrated between the sub-phenotypes of disease location and behavior for CD (results not shown).

	No. of individuals	**Log-likelihood analysis P-value**
Ulcerative colitis	249	4.22×10^{-7}
Extensive disease	102	1.7×10^{-7}
Left sided disease	147	0.0089
Crohn's disease	179	0.22

Table 7: Analysis of all combinations of 2-, 3-, 4- and 5-locus haplotypes using log-likelihood analysis using UC/control partition. Only haplotypes containing Tag 1 retains significance. D.f. – degrees of freedom, H1 – alternative hypothesis

Tags					Case	Control	Case+ Control	D.f	H1	P-value
1	2				503.62	436.52	968.29	3	56.3	3.63E-12
1	3				507.33	452.26	983.56	3	47.94	2.19E-10
1	4				505.16	480.54	999.17	3	26.94	6.06E-06
1	5				472.27	496.84	974.38	3	10.54	0.01
1	6				424.34	452.29	879.29	3	5.32	0.15
2	3				295.63	311.72	608.96	3	3.22	0.36
2	4				440.26	429.72	873.5	3	7.04	0.07
2	5				472.23	493.67	968.44	3	5.08	0.17
2	6				425.4	452.81	878.41	3	0.4	0.94
3	4				433.68	422.09	858.82	3	6.1	0.11
3	5				475.85	495.62	973.91	3	4.88	0.18
3	6				429.24	455.24	884.78	3	0.6	0.89
4	5				465.29	486.93	954.25	3	4.06	0.25
4	6				428.03	451.09	881	3	3.76	0.29
5	6				388.64	423.44	813	3	1.84	0.61
1	2	3			544.03	480.03	1055.67	7	63.22	3.42E-11
1	2	4			685.64	593.3	1313.2	7	68.52	2.94E-12
1	2	5			720.78	663.78	1415.47	7	62.28	5.28E-11
1	2	6			673.86	623.34	1325.78	7	57.16	5.56E-10
1	3	4			679.17	599.38	1307.53	7	57.96	3.85E-10
1	3	5			724.22	679.35	1429.82	7	52.5	4.65E-09
1	3	6			677.6	638.79	1340.9	7	49.02	2.25E-08
1	4	5			712.55	702.84	1429.8	7	28.82	0.000156
1	4	6			674.93	666.54	1355.88	7	28.82	0.000156
1	5	6			642.43	682.77	1331.02	7	11.64	0.11
2	3	4			469.76	465.45	939.96	7	9.5	0.22
2	3	5			512.41	538.71	1055.14	7	8.04	0.33
2	3	6			465.85	497.81	965.75	7	4.18	0.76
2	4	5			644.58	650.65	1300.66	7	10.86	0.14
2	4	6			610.61	613.42	1229.5	7	10.94	0.14
2	5	6			640.68	678.1	1322.71	7	7.86	0.35
3	4	5			638.17	644.06	1286.99	7	9.52	0.22
3	4	6			603.91	607.71	1215.81	7	8.38	0.30
3	5	6			644.83	679.46	1328.18	7	7.78	0.35
4	5	6			633.9	668.4	1305.81	7	7.02	0.43
1	2	3	4		715.11	625.13	1377.24	14	74	3.61E-10
1	2	3	5		760.74	706.22	1500.98	14	68.04	4.36E-09
1	2	3	6		713.65	664.71	1410.99	14	65.26	1.37E-08
1	2	4	5		889.11	813.5	1739.34	14	73.46	4.53E-10
1	2	4	6		854.91	775.99	1667.28	14	72.76	6.08E-10

1	2	5	6		883.46	846.54	1762.42	14	64.84	1.63E-08
1	3	4	5		882.85	820.8	1734.86	14	62.42	4.4E-08
1	3	4	6		847.81	784.08	1662.42	14	61.06	7.64E-08
1	3	5	6		886.76	861.71	1776.6	14	56.26	5.25E-07
1	4	5	6		875.87	883.46	1777.64	14	36.62	0.0008
2	3	4	5		674.05	685.84	1367.03	14	14.28	0.43
2	3	4	6		639.88	647.42	1294.67	14	14.74	0.39
2	3	5	6		680.84	721.43	1408.6	14	11.7	0.63
2	4	5	6		811.58	827.75	1650.99	14	23.32	0.06
3	4	5	6		805.34	822.06	1637.83	14	20.86	0.11
1	2	3	4	5	918.42	844.61	1802.25	31	78.44	5.49E-06
1	2	3	4	6	833.1	806.05	1728.67	31	79.1	4.42E-06
1	2	3	5	6	922.69	866.53	1845.63	31	72.8	3.21E-05
1	2	4	5	6	1048.9	988.59	2081.2	31	87.0	3.24E-07
1	3	4	5	6	1042.2	997.24	2076.4	31	73.92	2.29E-05
2	3	4	5	6	840.7	860.4	1714.6	31	27	0.67

Table 8: Comparison of direct haplotypic frequencies between UC and controls. We have selected for each of the respective 2, 3, 4 and 5-locus, the most significant haplotypes (see Table 4) for analysis. For each of the selected haplotypes, the inferred haplotypic frequencies for UC and controls were compared using Fisher's exact test. The most significant specific haplotypes are shown in the above table.

Haplotypes	**UC (n=249)**	**Controls (n=260)**	**P-value Odds ratio 95% CI**
1, 2 11	24.2%	8.9%	<0.00001 3.27 2.26-4.72
1, 2, 4 112	16.8%	7.8%	<0.00001 2.37 1.59-3.52
1, 2, 4, 6 1121	15.1%	6.4%	<0.00001 2.61 1.70-4.02
1, 2, 4, 5, 6 11211	7.6%	2.7%	0.0003 2.99 1.60-5.58

Chapter 7

Investigation into the contribution of Pregnane-X Receptor (PXR/NR1I2) gene and susceptibility to inflammatory bowel disease: Parallel allelic association study and gene-wide haplotype analysis

Abstract

Background: The Pregnane X-receptor gene (PXR/NR1I2) regulates an array of genes involved in the response to xenobiotics. Dysregulation of this gene may critically influence intestinal barrier defence and susceptibility to inflammatory bowel disease (IBD). Recent data in an Irish dataset have suggested strong associations between polymorphisms within the PXR/NR1I2 gene and IBD.

Aims: The allelic variants of rs1523127/-24381 of the PXR/NR1I2 gene previously implicated in the Irish dataset are now investigated in the Scottish dataset. A candidate gene-wide association study to assess in detail the contribution of this gene to disease susceptibility was also performed.

Methods: 387 ulcerative colitis (UC), 328 Crohn's disease (CD) patients and 338 healthy controls (HC) were studied. This study has >95% power to replicate previous association with the rs1523127/-24381 variant ($p < 0.05$). Five tagging SNPs (tSNPs) were selected by re-sequencing PXR/NR1I2 gene in 32 CEPH Caucasian trios, using a multimarker criterion, haplotype $r^2 > 0.80$ to predict all SNPs/haplotypes. Single marker, log-likelihood and genotype-phenotype analyses using the Montreal classification were used to test for association.

Results: No association was seen with rs1523127/-24381 SNP with UC, CD or IBD (A-allelic frequency 60.5% UC, 59.5% CD, 60.0% IBD and 60.6% HC; p= 0.96, 0.69 and 0.82 respectively). The homozygosity rates were 35.9%, 31.1%, 33.7% and 35.6% for UC, CD, IBD and HC respectively (p=0.91, 1.00 and 1.00 respectively). The log-likelihood analyses comparing overall haplotypic distribution of the 5 tSNPs, demonstrated no associations with UC, CD or IBD (p=0.90, 0.90 and 0.79 respectively). There were no associations observed between each of the 5 tSNPs with

UC, CD and IBD respectively. Genotype-phenotype analyses did not show any associations with the studied variants and haplotypes.

Conclusion: Germ-line variation of the PXR/NR1I2 gene does not have a major effect on susceptibility to IBD or disease phenotype in the Scottish population. However, epigenetic modification of this gene may be an important pathogenic event; and other genes in epithelial barrier function remain plausible candidate genes.

7.1 Introduction

Increasing evidence suggest a role in epithelial transporter and xenobiotic metabolising genes in the maintenance of intestinal barrier defence (Ho, Gaya, and Satsangi 2005). Much of the earlier interest was derived from data based on animal models(Panwala, Jones, and Viney 1998; Maggio-Price et al. 2002; Maggio-Price et al. 2005) and genetic studies involving the P-glycoprotein 170 pump (PgP 170) the gene product of MDR1 gene, as demonstrated in Chapters 6 and 7 in this thesis. The MDR1 gene is one of the many genes in the family of ATP-binding cassette (ABC) transmembrane transporters which are highly expressed in intestinal epithelial cells with very diverse functions – most pertinently the transport of drug substrates, endogenous steroid/cholestrol compounds, bile acids and other xenobiotics (Langmann et al. 2003). Other ABC transporters such as ABCC1-3 (multidrug resistance proteins 1-3) have also been demonstrated to have similar roles(Langmann et al. 2003).

Langmann and colleagues have recently further developed the argument for the contribution of defective detoxification in the development of IBD(Langmann et al. 2004). In this study, DNA microarray analysis involving non-affected colonic tissue of CD and UC, revealed a cluster of strongly down regulated detoxification genes (of the glutathione and sulfo-transferase family) and ABC transporters (including MDR1) in UC, together with the near complete loss of the transcriptional regulator pregnane X receptor (PXR). The expressions of MDR1 and PXR were both reduced in tissues of patients with UC in subsequent rt-PCR analysis in the same study. PXR is a nuclear hormone receptor that is ligand-activated by a large number of structurally and pharmacological diverse endogenous and exogenous compounds (including

pregnanes, corticosteroids, rifampicin and bile acids)(Staudinger et al. 2001; Chrencik et al. 2005; Schuetz et al. 2001; Willson and Kliewer 2002). PXR regulates the induction of many genes including CYP3A4 and the ABC transporter family genes (which includes MDR1). The function and expression of MDR1 is closely regulated by PXR(Kliewer, Goodwin, and Willson 2002).

Central to the findings of Langmann et al., is the suggestion that PXR play a pivotal role in the maintenance of epithelial barrier defence. It is postulated that dysregulated PXR expression and function leads to a widespread effect on the downstream detoxification genes therefore predisposes to increased risk of intestinal inflammation. On this basis, Dring and colleagues have hypothesised that germ-line variations of the PXR/NR1I2 gene may confer susceptibility to IBD. The authors performed a case-control study involving 422 patients with IBD (185 UC and 237 CD) and 350 healthy controls, using 8 candidate polymorphisms in this gene. Highly significant associations were demonstrated with UC, CD and IBD as a whole. This effect was most significant for the two individual SNPs in the promoter region of this gene; when compared between the IBD cohort and controls, -23585 (rs3814055) (p=0.000008; OR 1.62; 95% CI, 1.31-2.00) and -24381 (rs1523127) (p= 0.0002; OR, 1.50; 95% CI, 1.21-1.84).

In order to clarify the genetic contribution of the PXR/NR1I2 gene to the susceptibility to IBD, this candidate gene was resequenced and replication exercise of previous associations observed in the Irish dataset were performed. To provide a more complete assessment of the genetic contribution of this gene to disease susceptibility,

I utilised the candidate gene-wide strategy using a haplotype-tagging approach as successfully applied in the previous chapter.

7.2 Material and methods

Patients

A total of 387 patients with UC and 328 with CD were recruited from the Lothian region, Scotland, United Kingdom. The diagnosis of IBD was determined by standard clinical, radiological, endoscopic and histological criteria. Table 1 summarizes the clinical characteristics of patients studied. The median ages at diagnosis for UC and CD were 35.2 years (IQR 25.9-50.4) and 28.5 years (IQR 21.0-41.1) respectively. The ethnicity of our study population was Scottish-Caucasian. There were more males in the UC cohort (54.0%) than in CD (36.6%). 356 healthy controls which comprised of 102 actively recruited healthy subject and+ 254 blood donors from the Lothian region (50.8% males and age of recruitment 31.1 years, IQR 25.9-47.0).

Phenotypic assessment

The phenotypic data for both UC and CD were categorised using the new Montreal Classification(Silverberg et al. 2005). UC phenotype was classified by disease extent, severity and need for surgery. Extensive disease was defined as disease extending beyond splenic flexure (E3), left-sided colitis as disease extending to the splenic flexure (E2) and proctitis (E1), limited to the rectum using maximal colonoscopic evidence over time of follow-up. Patients who had developed acute severe attack of UC (satisfying the Truelove and Witts criteria) requiring intensive in-patient medical therapy were regarded to have severe UC (S3). Within the severe UC, a further subset

of patients who had failed to respond to medical therapy and consequently required surgery were categorized under the phenotype – need for surgery. Other phenotypic details such as smoking, family history, presence of primary sclerosing cholangitis (PSC) and other extra-intestinal manifestations were also recorded. CD was classified according to the new Montreal Classification of disease location (L1-L4), behaviour (B1-B3, with p-as perianal) and age of diagnosis (A1 <16 years, A2 17-40 years and A3 >40 years). I have presented disease behaviour data at 5-year following diagnosis.

Resequencing of the PXR/NR1I2 gene and the selection of tSNPs

The exons, promoter region and intronic boundaries were re-sequenced in 16 unrelated Centre d'Etude du Polymorphisme Humain (CEPH) individuals by using 10 randomly spaced amplicons across the PXR/NR1I2 gene. All SNPs discovered were resequenced in 32 CEPH trios. Haplotypes and their respective frequencies were estimated and constructed from all genotyped SNPs using the partition-ligation expectation-maximization algorithm(Zhang et al. 2004). A set of tSNPs were then selected to 'tag' the majority of the constructed haplotypes. The selection criteria were described in Chapter 7.

Statistical analysis

The statistical methods to compare allelic and genotypic frequencies were described in Chapter 6. The log-likelihood ratio tests to test for association, and the contingency tests between constructed haplotypes were described in detail in Chapter 7. A power calculation was undertaken using previous reported association with the rs1523127/-24381 variant in the Irish dataset using a genotypic model of analysis (i.e. based on a

2 by 3 contingency table). Based on the size of the IBD cohort, this study has a 98% power to replicate this association at the level of $p < 0.05$.

7.3 Results

Re-sequencing of PXR/NR1I2 gene

Re-sequencing of the exons, promoter region and intronic boundaries in 16 unrelated CEPH white individuals over a region spanning 7.4 kB revealed 15 SNPs with a minor allele frequency of greater 10%. These SNPs were further genotyped in 32 CEPH trios. 26 haplotypes with inferred frequencies of >0.5% were used for further selection of tSNPs. Using 15 SNPs with high minor allele frequency, a set of 5 tSNPs (rs1523127, rs2461823, rs7643645, rs1464603 and rs2472682) were identified that provide a coefficient of determination, haplotype r^2 of 0.80 in predicting all the known SNPs/haplotypes. These tSNPs were genotyped in 387 UC, 328 CD patients and 338 healthy controls. All genotype data for these groups were in Hardy-Weinberg equilibrium.

Single variant analyses and replication of previously implicated candidate polymorphisms (Table 2)

The rs3814055 (-23585) and rs1523127 (-24381) SNPs were in strong linkage disequilibrium, with an r2-value of 0.96. Both these SNPs were strongly associated with IBD in Dring's study. In view of this linkage magnitude, only one of the variants can be studied without any loss of information of the other and the rs1523127/-24381 variant was genotyped in the Scottish dataset. We did not observe any associations between this SNP and susceptibility for IBD and controls in our study (A-allelic frequencies, p=0.82, odds ratio 0.97, 95% CI 0.81-1.17; and AA-genotype frequencies

p=1.00, odds ratio 0.99, 95% CI 0.66-1.49) (Table 2). No associations were seen with either UC or CD respectively.

The rs6785049 (-7635) variant which was also significantly associated with IBD in Dring's study was in strong linkage with a further tSNP used in this study (rs2472682) with an r^2-value of 0.86. In the Scottish dataset, this SNP (rs2472682) was also not associated with IBD (T-allele, p=0.97, OR 1.00, 95% CI 0.82-1.22 and TT-genotype, p=0.82, OR 0.96, 95% CI 0.62-1.47). There were no significant differences between UC, CD and healthy controls respectively for the other respective tSNPs (Table 2).

Log-likelihood analyses (Table 3)

Providing the more robust assessment of the contribution of the genetic variations of this gene to disease susceptibility, the log-likelihood analysis which examine the differences between the overall haplotypic distribution between case and control (which makes a likelihood assessment that includes statistical uncertainty in phase estimation), showed no associations with UC, CD or IBD (p=0.90, 0.90 and 0.78 respectively). On direct haplotypic comparisons between case and controls (total of 32 haplotypes), we did not observe any significant differences between UC and CD, with controls. We presented data on the four commonest haplotypes and showed no significant differences in the groups of UC, CD and HC (Table 3b).

Subphenotypic analyses using Montreal Classification

In addition, on subphenotypic analysis using the Montreal classification, no associations were observed for both single variants and haplotypes for age of

diagnosis, disease location and behaviour for CD; and disease extent, severity and surgery for UC.

Comparisons between Scottish and Irish study populations (Table 4)

Using the rs1523127/-24381 SNP, it is noteworthy that significant differences in the allelic and genotype frequencies exist between the Scottish and Irish healthy control populations (A-allele frequencies: p=0.03, OR 1.27, 95% CI 1.02-1.58 and AA-genotype frequencies: p=0.01, odds ratio 1.75, 95% CI 1.12-2.72). The minor allelic frequency of our control population was 40% compared with the identical frequencies of 39% for CEPH population obtained from HapMap Project (http://www.hapmap.org) and our initial sequencing of the 32 CEPH trios respectively. This contrasted with the higher frequency, 45% observed in the Irish control population. These differences were very much less marked when comparing the allelic and genotypic frequencies between the Scottish and Irish patient populations (A-allele frequencies: p=0.05, OR 1.20, 95% CI 1.00-1.44 and AA-genotype frequencies: p=0.17, odds ratio 1.33, 95% CI 0.90-1.98)

7.4 Discussion

The PXR/NR1I2 gene represents an attractive candidate gene in determining susceptibility to IBD. The recent associations between candidate polymorphisms within this gene and IBD in the Irish population are exciting and bear further examination. In this study, I have attempted to replicate the previous association by Dring et al. in the Scottish population. Secondly, we have proceeded to use a more exhaustive method to assess the overall contribution of this gene to disease susceptibility by using a multimarker haplotype-tagging approach that captures the

genetic variations of this gene. In contrast to the study by Dring et al., I did not replicate the previous association of the rs1523127, -24381 variant with IBD (UC and CD respectively). In addition, our haplotypic analyses did not show any association.

A number of issues deserve further discussion in view of the apparent discordant findings between this and Dring et al.'s study. Firstly, do these data reflect true genetic heterogeneity between the Irish and Scottish populations? The Scottish population is traditionally characterised by low rates of admixture and considered to be ethnically close to the Irish. This has recently been demonstrated in the similar low rates of NOD2/CARD15 gene mutations in these populations as compared to other European and North American studies (Arnott et al. 2004a). Indeed, the NOD2/CARD15 data have led to the search of common founder effects in the Scottish, Irish and other North European populations(Gaya et al. 2006). Thus, the argument in favour of genetic heterogeneity is intuitively considered to be minimised.

Closer examination of the present data reveals that the discrepancies between the conclusions arise due to differences not in the comparison between patient groups, but between controls in Ireland and Scotland. It is of great interest to note that the allelic and genotypic frequencies of the rs1523127/-24381 SNP (which was studied in both the Irish and Scottish datasets) were significantly different between the control populations of Ireland and Scotland. The allelic frequencies of this variant was 40% in the Scottish and 45% in the Irish control population respectively (p=0.03, odds ratio 1.24, 95% CI 1.03-1.49). The data for CEPH population obtained from HapMap Project, our initial genotype data in 32 CEPH trios and the white Caucasian populations in the study by Zhang et al. (for which the natural allelic variants of this

gene were originally characterised) were 39%, 39% and 40% respectively (www.hapmap.org)(Zhang et al. 2001). In fact, data from both Irish and Scottish-IBD cohorts revealed also minor allelic frequencies of 38% and 40%. Thus it may be possible that cryptic stratification of the control population may belie the positive results demonstrated in the Irish study.

What other possible explanations exist? It is widely accepted that phenotypic heterogeneity e.g. composition of the IBD cohort can sometimes explain apparent inconsistent results across different study groups with the most relevant examples being the NOD2/CARD15 gene and IBD5 risk haplotype(Ahmad et al. 2002; Cuthbert et al. 2002; Noble et al. 2005; Vermeire et al. 2005; Torok et al. 2005; Armuzzi et al. 2003; Giallourakis et al. 2003; Mirza et al. 2003; Negoro et al. 2003). The Irish dataset suggested an association with several subphenotypes in IBD (CD-ileal and colonic disease location, UC- pancolitis). Our cohort has been the subject rigorous phenotypic assignment using the new Montreal Classification, including disease behaviour, disease severity, age of onset and smoking habit; and it is pertinent that no associations were observed both from single marker or haplotypic analyses. Specifically, we did not replicate the associations previously suggested with disease location in CD and extent in UC.

Thirdly, the power of the sample size has emerged as central factor in determining the validity of a replication study in complex genetics. The difficulties encountered, are well-demonstrated in the studies of DLG5 gene(Stoll et al. 2004) and OCTN1/2 gene(Peltekova et al. 2004) in inflammatory bowel diseases, and these have been reviewed in detail by Trinh and Rioux(Trinh and Rioux 2005). The current study is

adequately powered to detect the previous associations demonstrated in Dring's study, assuming the effect size of rs1523127/-24381 SNP (>95% at a $p<0.05$). It is therefore unlikely that the findings represent a type 2 error. In addition, the haplotype r^2 of 0.80 reflected a predictable state of the tagged variants by the haplotypes defined by the selected five tSNPs and thus, a statistically powerful method to detect even a weak signal or effect from this locus or gene. This negative this gene-wide haplotype analysis therefore sets a statistical limit to the importance of this gene in conferring susceptibility to IBD in our population.

How robust is the current approach in detecting association in a genetic model between different populations? Ahmadi and colleagues recently demonstrated the applicability of this approach in a proof-of-concept study by assessing the contribution of 55 genes in 2 populations by using a set of tSNPs selected using a multiple-marker criterion (haplotype r^2) that selects tSNPs independently of any underlying block structure, ignoring heuristically defined block boundaries(Ahmadi et al. 2005). Extensive empirical evaluations, including a direct assessment of association with candidate functional SNPs in a new, larger population sample, validated the performance of these tagging SNPs and confirmed their utility for linkage-disequilibrium mapping in pharmacogenetics. Similarly, De Bakker et al. have shown that such approach is economical and efficient, with very little loss of power compared to traditional single pair-wise methods(de Bakker et al. 2005).

Notwithstanding the negative findings of this study, dysregulation of PXR/NR1I2 gene leading to the development of gut inflammation, remains plausible and could be influenced through other ways for example, epigenetic mechanisms or a situation

where multiple susceptibility genes are necessary for biological effect, thus providing a possible explanation for the wide spectrum of disease location and behaviour in IBD. The PXR/NR1I2 gene shares a considerable overlap with the Constitutive Androstane Receptor (CAR) in the regulation of a number of epithelial transporter and xenobiotic-metabolizing enzymes, and the contribution of the latter gene has not yet been studied. The number of target genes for both PXR/NR1I2 and CAR is considerable (more than 20) including the Cytochrome P450 genes and ABC transporters. Germ-line variations of one of the ABC transporters, the ABCB1/MDR1 gene has been shown to confer susceptibility to IBD (in particular UC) in several study populations(Schwab et al. 2003b; Brant et al. 2003; Ho et al. 2005; Ho et al. 2006b; Urcelay et al. 2006; Potocnik et al. 2004). It is not inconceivable that all the other genes which are regulated by PXR/NR1I2 and CAR may all be good independent candidate genes; or in conjunction with PXR/NR1I2 and CAR. In such circumstances, gene-gene interactions or regulatory SNPs controlling gene expression may be pertinent, and by genotyping the entire 'family' of xenobiotic-metabolising enzymes can we reliably established the genetic contribution of these genes (and indeed PXR/NR1I2).

In conclusion, genetic variations in PXR/NR1I2 gene do not have a major effect on susceptibility to IBD in the Scottish population. Further studies in different ethnic and study populations are necessary to provide a clearer role for this gene in IBD.

Table 1a: Demographics and clinical characteristics of UC study population (n=387). Disease location (E1 – proctitis, E2 – left sided disease and E3 – extensive colitis) and severity (S3- severe UC as defined by Truelove and Witts criteria) are defined using the Montreal Classification(Silverberg et al. 2005).

Characteristics	UC (n=387)
Sex (M/F)	209/178
Age of onset (years)	35.2 (25.9-50.4)
Age of onset <16 years	11 (2.8%)
Current smoker Ex-smoker Never smoked Unknown	43 (11.1%) 154 (39.8%) 188 (48.6%) 2 (0.55)
Disease extent E1 E2 E3 Unknown Disease severity S3 Surgery for S3	 70 (18.1%) 165 (42.6%) 136 (35.1%) 16 (4.1%) 124 (32.0%) 79 (30.4%)
Extra-intestinal manifestations Primary Sclerosing Cholangitis	45 (11.6%) 7 (1.8%)
Drug therapy Azathioprine	 107 (27.6%)

Table 1b: Demographics and clinical characteristics of patients with CD. Disease location (L1-Ileal, L2-colonic, L3-ileocolonic, L4-upper GI); behaviour (B1-inflammatory, B2-stricturing, B3-penetrating and p-perianal) and age of onset (A1-<16 years, A2- 17-40 years and A3->40 years) were defined according to the Montreal Classification(Silverberg et al. 2005).

Characteristics	Crohn's disease (n=328)
Sex (M/F)	120/208
Smoking	
Current	88 (26.8%)
Ex-smoker	136 (41.5%)
Never	93 (28.4%)
Unknown	11 (3.3%)
Age of onset, years	28.5
Median (+IQR)	(21.0-41.1)
Age of diagnosis	
A1	28 (8.5%)
A2	215 (65.6%)
A3	85 (25.9%)
Drug therapy	
Infliximab	45 (13.7%)
Azathioprine	135 (41.2%)
Disease Location	
L1	108 (33.0%)
L1+L4	7 (2.2%)
L2	108 (33.0%)
L2+L4	4 (1.2%)
L3	77 (20.4%)
L3+L4	15 (4.6%)
L4	13 (4.0%)
Unknown	5 (1.5%)
Disease behaviour	
B1	171 (52.1%)
B1p	40 (12.2%)
B2	41 (12.5%)
B2p	4 (1.2%)
B3	54 (16.5%)
B3p	9 (2.7%)
Unknown	9 (2.7%)
Surgery	216 (65.8%)
Extra-intestinal manifestations	118 (43.1%)

Table 2: Allele and genotype frequencies of five tSNPs in UC, CD and healthy controls

dbSNP ID Position	Allele (1/2)	UC	CD	IBD	HC	UC vs. HC 1 vs. 2 P-value odds ratio 95% CI	UC vs. HC 1/1 vs. 2/2 P-value odds ratio 95% CI	CD vs. HC 1 vs. 2 P-value odds ratio 95% CI	CD vs. HC 1/1 vs. 2/2 P-value odds ratio 95% CI	IBD vs. HC 1 vs. 2 P-value odds ratio 95% CI	IBD vs. HC 1/1 vs. 2/2 P-value odds ratio 95% CI
rs1523127	AA	139 (35.9%)	102 (31.1%)	241 (33.7%)	119 (35.6%)	0.96	0.91	0.69	1.00	0.82	1.00
	AG	190 (49.1%)	186 (56.7%)	376 (52.6%)	167 (50.0%)	0.99	0.97	0.95	1.03	0.97	0.99
120983729	GG	58 (15.0%)	40 (12.2%)	98 (13.7%)	48 (14.4%)	0.80-1.22	0.61-1.52	0.76-1.19	0.62-1.69	0.81-1.17	0.66-1.49
	A	468 (60.5%)	390 (59.5%)	858 (60.0%)	405 (60.6%)						
	G	306 (39.5%)	266 (40.5%)	572 (40.0%)	263 (39.4%)						
rs2461823	CC	152 (40.9%)	132 (40.2%)	284 (40.6%)	119 (35.8%)	0.38	0.71	0.46	0.80	0.38	0.66
	CT	175 (47.0%)	157 (47.9%)	332 (47.4%)	174 (52.4%)	1.11	1.11	1.09	1.10	1.09	1.10
121002815	TT	45 (12.1%)	39 (11.9%)	84 (12.0%)	39 (11.7%)	0.88-1.37	0.68-1.81	0.87-1.37	0.66-1.82	0.90-1.32	0.71-1.70
	C	479 (64.4%)	421 (64.2%)	900 (64.3%)	412 (62.0%)						
	T	265 (35.6%)	235 (35.8%)	500 (35.7%)	252 (38.0%)						
rs7643645	TT	143 (39.7%)	128 (43.2%)	271 (41.3%)	128 (38.5%)	0.79	0.90	0.82	0.62	0.73	0.91
	TC	172 (47.8%)	120 (40.5%)	292 (44.5%)	168 (50.6%)	1.04	1.04	1.04	0.87	1.04	0.96
121008187	CC	45 (12.5%)	48 (16.2%)	93 (14.2%)	42 (12.6%)	0.84-1.29	0.64-1.69	0.82-1.30	0.54-1.42	0.86-1.26	0.63-1.46
	T	458 (63.6%)	376 (63.5%)	834 (63.6%)	424 (62.7%)						
	C	262 (36.4%)	216 (36.5%)	478 (36.4%)	252 (37.3%)						
rs1464603	AA	172 (45.9%)	153 (46.6%)	325 (46.3%)	167 (49.4%)	0.61	1.00	0.72	1.00	0.62	1.00
	AG	159 (42.5%)	138 (42.1%)	297(42.3%)	130 (38.5%)	0.94	0.98	0.96	1.01	0.95	0.99
121009039	GG	43 (11.5%)	37 (11.3%)	80 (11.4%)	41 (12.1%)	0.75-1.17	0.61-1.58	0.76-1.21	0.62-1.67	0.78-1.15	0.65-1.52
	A	503 (67.2%)	444 (67.7%)	947 (67.5%)	464 (68.6%)						
	G	245 (32.8%)	212 (32.3%)	457 (32.5%)	212 (31.4%)						
rs2472682	TT	42 (11.0%)	35 (11.1%)	77 (11.0%)	39 (11.7%)	0.65	1.00	0.63	0.70	0.97	0.82
	TG	177 (46.2%)	129 (40.8%)	306 (43.8%)	140 (42.2%)	1.06	1.00	0.94	0.90	1.00	0.96
121015342	GG	164 (42.8%)	152(48.1%)	316(45.2%)	153(46.1%)	0.84-1.32	0.62-1.64	0.74-1.19	0.54-1.50	0.82-1.22	0.62-1.47
	T	261 (34.1%)	199 (31.5%)	460 (32.9%)	218 (32.8%)						
	G	505 (65.9%)	433 (68.5%)	938 (67.1%)	446 (67.2%)						

Table 3a: Log-likelihood significance between the common haplotypes of PXR/NR1I2 gene haplotypes and IBD/UC/CD. Significance for association is calculated using the test statistic 2*(ln(L case)+ln(Lcontrol)-ln(Lcase/Lcontrol)), which has a χ^2 distribution with n-1 degrees of freedom (where n=number of inferred haplotypes)

	Log-likelihood P-value
IBD vs. controls	0.79
UC vs. controls	0.90
CD vs. controls	0.90

Table 3b: Comparison of 4 common haplotype frequencies between case and controls.

Haplotype	**UC (%)**	**CD (%)**	**HC (%)**	**UC vs. HC P-value OR 95% CI**	**CD vs. HC P-value OR 95% CI**
12212	14.2	15.2	14.1	1.00 1.01 0.75-1.36	0.64 1.09 0.80-1.47
21121	9.6	10.0	9.3	0.86 1.04 0.73-1.49	0.64 1.11 0.77-1.59
22121	6.2	5.6	7.1	0.53 0.87 0.57-1.31	0.31 0.78 0.50-1.22
22212	5.9	7.5	7.1	0.39 0.83 0.54-1.26	0.83 1.06 0.70-1.60

Table 4a: Comparisons of allele and genotypic frequencies SNP, rs1523127/-24381 (implicated SNP in the study by Dring et al.) between Scottish and Irish populations

rs1523127 Scots	**AA**	**AC**	**CC**	**A**	**C**	**A vs. C P-value OR 95% CI**	**AA vs. CC P-value OR 95% CI**
UC (n=387)	139 (35.9%)	190 (49.1%)	58 (15.0%)	468 (60.5%)	306 (39.5%)	0.96 0.99 0.80-1.23	0.91 0.97 0.61-1.52
CD (n=328)	102 (31.1%)	186 (56.7%)	40 (12.2%)	390 (59.5%)	266 (40.5%)	0.69 0.95 0.76-1.19	1.00 1.03 0.63-1.69
IBD (n=715)	241 (33.7%)	376 (52.6%)	98 (13.7%)	858 (60.0%)	572 (40.0%)	0.82 0.97 0.81-1.18	1.00 0.99 0.66-1.50
HC (n=334)	119 (35.6%)	167 (50.0%)	48 (14.4%)	405 (60.6%)	263 (39.4%)		
Irish UC (n=176)	-	-	-	226 (64.2%)	126 (35.8%)	0.003 1.49 1.14-1.94	-
CD (n=223)	-	-	-	287 (64.3%)	159 (35.7%)	0.001 1.50 1.17-1.92	-
IBD (n=399)	164 (41.1%)	185 (46.4%)	50 (12.5%)	513 (64.3%)	285 (35.7%)	0.0002 1.50 1.21-1.84	0.003 2.31 1.50-3.56
HC (n=336)	108 (32.2%)	152 (45.2%)	76 (22.6%)	368 (54.8%)	304 (45.2%)		

Table 4b: Comparisons of allele and genotypic frequencies SNP, rs1523127/-24381 in IBD and control populations of the Irish and Scottish populations.

Comparison between these 2 groups		Allelic frequency A vs. C	Genotype frequency AA vs. CC
Scots IBD	Scots Control	0.82 0.97 0.81-1.18	1.00 0.99 0.66-1.50
Irish IBD	Irish Control	0.0002 1.50 1.21-1.84	0.003 2.31 1.50-3.56
Scots IBD	Irish Controls	0.03 1.24 1.03-1.49	0.005 1.73 1.19-2.52
Scots Controls	Irish Controls	0.03 1.27 1.02-1.58	0.01 1.75 1.12-2.72
Irish IBD	Scots Controls	0.16 1.17 0.94-1.44	0.24 1.32 0.83-2.10
Irish IBD	Scots IBD	0.05 1.20 1.00-1.44	0.17 1.33 0.90-1.98

Chapter 8

Gene-wide haplotypic analysis demonstrates association between ATP-Binding cassette 3/ Multidrug Resistance Protein 3 (ABCC3/MRP3) gene and inflammatory bowel disease

Abstract

Background/Aims : Increasing evidence implicates the dysregulation of intestinal epithelial defence mechanisms and xenobiotic metabolism in inflammatory bowel disease (IBD). Downregulation of a group of efflux transporters/xenobiotic metabolizing genes including *multidrug resistance 1, ABCB1/MDR1* and the transcriptional regulator, *Pregnane X-receptor* (PXR) in IBD have been demonstrated ; inherited variations of both genes have been associated with IBD. The *ATP-binding cassete sub-group C, family 3* (ABCC3) gene encodes multidrug resistance protein 3 (MRP3) an efflux epithelial transporter pump which is highly expressed in the gut, sharing close homology with ABCB1/MDR1.

Methods : A candidate gene-wide haplotypic analysis of the ABCC3 gene in a total of 1197 patients with IBD (669 UC, 528 CD) and 613 healthy controls in two independent case-control cohorts of Scottish and Swedish descent were utilised. Eight tagging SNPs (tSNPs) were selected with a minimum haplotype r^2 of 0.80 represent the haplotypic variations of this gene. Single marker, log-likelihood and genotype-phenotype analyses using the Montreal classification were used to test for association.

Results : Using log-likelihood analysis, haplotypic variations in ABCC3 gene were associated with IBD, UC and CD in Scottish (p=0.00046, 0.004 and 0.001 respectively) and Swedish cohorts (p=0.001, 0.003 and 0.004 respectively). Genotype-phenotype associations with inflammatory behaviour phenotype (B1), ileo-colonic disease (L3) in CD and extensive UC (p=0.005, 0.02 and 0.04 respectively) were present. The possession of described risk allele of PXR gene (rs1523127) increased the associaton with IBD (p=0.005) whereas no epistatic interactions were evident with ABCB/MDR1 variants and smoking habit. Significant single marker association was observed in 4 out of 8 tSNPs within the Scottish IBD, UC and CD

patients (maximal association tSNP6 rs879459 p=0.006, OR 1.52, 95% CI 1.14-2.04 ; tSNP6, rs879459, p=0.0008, odds ratio 1.72, 95% CI 1.25-2.38 ; and tSNP3, rs739921, odds ratio 1.41, 95% CI 1.11-1.78 respectively).

Conclusions : These findings provide strong evidence for a role for ABCC3/MRP3 in IBD susceptibility in Northern Europe, the contribution is independent to that of the MDR1/ABCB1 gene.

8.1 Introduction

As discussed earlier, recent genetic studies have now implicated the *organic cation transporters 1 and 2* (OCTN1/2)(Peltekova et al. 2004), *drosophila large gene 5* (DLG5)(Stoll et al. 2004), *multidrug resistance 1* (ABCB1/MDR1)(Ho et al. 2005; Ho et al. 2006b), *pregnane X-receptor* (PXR/NR1I2)(Dring et al. 2006) and *myosin IX9* (MYOB9) genes in the susceptibility to IBD. The functions of all these genes are associated with the maintenance of intestinal epithelial integrity and barrier defence. In addition, the NOD2/CARD15 gene which represents the most widely replicated genetic association with CD(Ogura et al. 2001b; Hugot et al. 2001a), is associated with defective defensin production thereby compromising the gut barrier defence(Wehkamp et al. 2005; Lala et al. 2003). Following the recent microarray data in human IBD by Langmann and colleagues, the critical importance of detoxification genes and epithelial efflux transporter proteins (in the ATP-binding cassete family, ABC transporters) in IBD are re-emphasised as evident from the significant down-regulation of *Pregnane X-receptor*, the key transcriptional regulator of most of the xenobiotic and ABC proteins (including ABCB1/MDR1 gene) (Langmann et al. 2004) (Kliewer, Goodwin, and Willson 2002). As shown in the preceeding chapter, variations of the PXR gene is not implicated in disease susceptibility in the Scottish population. Given the previous positive associations with ABCB1/MDR1 gene with UC in our dataset, I hypothesized that other efflux transporters that share a close homology with P-glycoprotein 170 (the gene product of ABCB1/MDR1) and also controlled by transcriptional regulator PXR, namely the ABC transporters may contribute to disease susceptibility.

In this present study, I have studied in detail the contribution of the ABCC3 (*ATP-binding cassette, sub-family C (MRP3), member 3, multidrug resistance protein 3*, MRP3) which was implicated in Langmann's study in IBD. The ABCC3 gene is 56 kb in length and maps to chromosome 17q21 region. This gene encodes for the *multi-resistant protein 3 transporter* pump (MRP3) which mediates the basolateral efflux of sulfated bile salts, lipophilic substances conjugated with glucuronate, sulphate or glutathione, across the basolateral membrane of polarized cells and glucuronide-conjugated organic anions(Kullak-Ublick, Stieger, and Meier 2004). MRP3 is highly expressed in cholangio-, entero- and colonocytes. In animal models, MRP3 is poorly expressed in stomach and upper small intestines with marked up-regulation in the colon and to a lesser extent, the ileum (Rost et al. 2002; Mutch et al. 2004). To determine whether variations of ABCC3 gene are associated with disease susceptibility, I employed a 'gene-wide' haplotype-tagging strategy in a large Scottish dataset and sought to replicate potential positive findings in a further Swedish dataset.

8.2 Material and Methods

In this study, a total of 387 patients with UC and 328 with CD recruited from the Lothian region, Scotland, United Kingdom as previously described were genotyped. The diagnosis of IBD was determined by standard clinical, radiological, endoscopic and histological criteria. Table 1 summarizes the clinical characteristics of patients studied. The median ages at diagnosis for UC and CD were 35.2 years (IQR 25.9-50.4) and 28.5 years (IQR 21.0-41.1) respectively. The ethnicity of our study population was Scottish-Caucasian. There were more males in the UC cohort (54.0%) than in CD (36.6%). 338 healthy controls which comprised of 102 actively recruited healthy subject and 254 blood donors from the Lothian region (50.8% males and age

of recruitment 31.1 years, IQR 25.9-47.0). In the Swedish dataset, 284 and 192 individuals with UC and CD respectively were recruited from hospitals in the Stockholm County were assessed. The Swedish controls (male : female – 126/151 healthy volunteers recruited from Karolinske Univeristy Hospital and day case surgery patients ; median age 49.61 year, IQR 38.45-62.67). The demographics and clinical details of these patients are shown in Table 2. These patient groups and controls have been previously described in previous studies(Torkvist et al. 2006b; Noble et al. 2005; Ho et al. 2005).

Haplotype tagging of ABCC3 gene

The ABCC3 gene was re-sequenced in 16 unrelated Centre d'Etude du Polymorphisme Humain (CEPH) individuals by using randomly spaced amplicons across the ABCC3 gene from position -49186865 to -49243698 in chromosome 17. All SNPs discovered were further genotyped in 32 CEPH trios. Haplotypes and their respective frequencies were estimated and constructed from all genotyped SNPs using the partition-ligation expectation-maximization algorithm(Zhang et al. 2004). We used a haplotype r^2 method described by Weale and Goldstein to select the current tSNPs(Weale et al. 2003; Goldstein et al. 2003). The criteria for the choice of tSNPs were i) average r^2 weighted by allele frequency between the tSNPs and all SNPs were $\geq$0.80 and ii) minimum r^2 between the tSNPs and each individual SNPs in the gene was 0.80. All genotype data was entered into the TagiT v.3 (criterion five) www.genome.duke.edu/research/centers/pg2) with a minimum haplotype r^2 of 0.80 to select the tSNPs.

Statistical analysis

Case and control frequencies were compared using a 2X2 contingency table and Fisher's exact test. Odd ratios (OR) were given with 95% confidence intervals and 2-sided P-values using the Graph Pad Instat programme (Graph Pad Software, San Diego, USA). Haplotype frequencies of the ABCC3 tSNPs were inferred using the expectation-maximization (EM) algorithm (Zhao, Curtis, and Sham 2000). Haplotypic associations with disease were assessed by using log-likelihood ratio analysis implemented in the EH and PM programs as discussed in previous chapters (Zhao, Curtis, and Sham 2000; Qin, Niu, and Liu 2002). We measured the linkage disequilibrium between SNPs using r^2 values(Hill W.G. and Robertson A 1968).

Further haplotypic analyses were performed using the Montreal Classification as the partition for log-likelihood analyses (e.g. we compared the haplotypes of patients with extensive UC with controls)(Silverberg et al. 2005). The similar analyses were also performed in stratified sets within the case group according to smoking status, sex and allelic variations of the recently cited ABCB1/MDR1 (rs3789243) and PXR gene (rs1523127) (only in the Scottish cohort) (Ho et al. 2006b; Ho et al. 2006c; Dring et al. 2006). In addition to this, we investigated the relative estimated constructed haplotype frequencies of the ABCC3 gene in the Scottish, Swedish and controls, using the chi square method (2 x 2 tables).

8.3 Results

In total, 1197 patients with IBD (669 UC, 528 CD) and 613 healthy controls were genotyped with 8 tSNPs capturing the haplotypic variations of the ABCC3 gene in this current study. All tSNPs satisfied the Hardy-Weinberg equilibrium. The average genotyping success was 95%.

Single variant and haplotypic associations of ABCC3 gene in the Scottish cohort

Significant association with IBD was demonstrated with 4 out of 8 tSNPs within the Scottish cohort (Table 4), with the most significant association ascribed to tSNP6 (rs879459, p=0.0058, odds ratio 1.52, 95% CI 1.14-2.04). Within the Scottish cohort, associations were observed for both UC (maximal association with tSNP6, rs879459, p=0.0008, odds ratio 1.72, 95% CI 1.25-2.38) and CD (tSNP3, rs739921, odds ratio 1.41, 95% CI 1.11-1.78). Using the log-likelihood ratio analysis which tests the association between the inferred haplotypes using disease/control partition (using EH+ which makes a likelihood assessment that includes statistical uncertainty in phase estimation), I detected a strong association of haplotypes with IBD in the Scottish dataset, with a p-value of 0.00046 (using 44 degrees of freedom, based on the cut-off of 45 haplotypes with an inferred frequency of >0.5%). Haplotypic associations of the ABCC3 were more strongly associated with CD (p=0.001) compared with UC (p=0.004).

Genotype-phenotype association of ABCC3 gene in the Scottish cohort

I performed further genotype-phenotype haplotypic analyses using the Montreal Classification partition, with significant associations demonstrated with the phenotypes of inflammatory behaviour (B1-at latest follow-up) (0.005), ileal-colonic CD (p=0.02) and extensive UC (p=0.04). We have further stratified our current dataset using previously implicated tSNPs of the multidrug resistance gene, *ABCB1/MDR1*, rs3789243; rs1523127/-24381 of the Pregnane X-receptor gene, *PXR/NR1I2* and smoking habit to investigate for interactions between these factors. It is of interest to note that the carriage of risk allele tSNP rs1523127/-24381 *PXR/NR1I2* retained a highly significant association of ABCC3 tSNPs with IBD (A-allele carriage p=0.005 vs. G-allele carriage p=0.06) (Table 5b). In contrast, there were no interactions with ABCB1/MDR1 or smoking habit in the Scottish dataset. Formal single variant analyses of the 8 tSNPs with the variants of PXR/NR1I2 and ABCC3 as described above, did not reveal significant interactions between these alleles and disease susceptibility in IBD, UC and CD (data not shown).

Haplotypic association of ABCC3 gene and genotype-phenotype analyses in the Swedish cohort

In the Swedish dataset, no significant associations were demonstrated at the single locus level using the tSNPs. Nevertheless, there remains a highly significant overall haplotypic association, with a p-value of 0.004 for IBD. Haplotypic associations were both significant for UC and CD (p=0.003 and 0.004 respectively). It is of interest to note that the phenotypic associations with inflammatory behaviour (B1) and ileo-colonic CD (L3) were retained (p=0.01 and 0.03 respectively) similar to Scottish dataset.

Comparison of constructed haplotype frequencies between case and controls

In Table 6, we present the ten common inferred haplotype frequencies in the current dataset. Within this subset, only one haplotype (22121222) was significantly associated with CD (p= 0.02, odds ratio 1.76, 95% CI 1.11-2.82). A total of 256 haplotypes were inferred from 8 tSNPs with 45 haplotypes of frequency >0.5% accounting for 85% of the inferred haplotypes. This suggests that the observed overall haplotypic association as evident from log-likelihood analyses may be driven by several different haplotypes collectively.

Given that our current tSNP selection strategy does not rely on heuristically defined block boundaries, we re-tested our tSNPs by using the available HapMap phase II (HapMap data release 21, July 2006 National Centre for Biotechnology Information [NCBI] build 35 assembly, dbSNP build 125) data to determine whether these tSNPs reside within a defined haplotype block in ABCC3 gene. Based on the solid spine LD analysis, the ABCC3 gene is composed of 5 haplotype blocks (4-10 kb) (Figure 1). On these criteria, each defined haplotype block was represented by one or more of our current tSNP, therefore suggesting that the ABCC3 gene is well-represented but also the 'causal' variant may reside anywhere along the length of this gene.

8.4 Discussion

The present data provides strong novel evidence for a role in the ABCC3 gene in determining susceptibility to inflammatory bowel disease. By using a gene-wide haplotype approach which captures the majority of the haplotypic variations of this gene, the association with IBD was most significant within the Scottish dataset (p=0.00046), with both the sub-phenotypes of UC and CD implicated (p=0.004 and 0.001 respectively). Using log-likehood analyses on the haplotypic level, this finding was replicated in a separate Swedish cohort (p=0.001, 0.004 and 0.003 for IBD, CD and UC respectively). Detailed phenotypic sub-analyses again revealed consistent sub-phenotypic associations with inflammatory behaviour and ileo-colonic disease in CD in both Scottish and Swedish cohorts. This present study therefore demonstrate independent associations in two Northern European cohorts.

The involvement of ABCC3 gene now emphasises the importance of ABC efflux transporters in the maintanence of gut barrier defence and susceptibility to IBD. Within this group, ABCB1/MDR1, CFTR and PXR have recently been implicated. I have characterised the gene expression pattern of the entire ABC transport protein and interestingly demonstrate that the expression of ABCC3/MRP3 gene is lowest distally (with an increasing gradient, similar to ABCB1/MDR1). Genetic mutations within the ABC transporters family are known to result in human diseases, for examples MRP2/ABCC2 (Dubin-Johnson's syndrome)(Toh et al. 1999), ABCG5/8 (sitosterolaemia)(Lu et al. 2001) and most strikingly CFTR gene predisposing to cystic fibrosis(Gadsby, Vergani, and Csanady 2006). If variations of the ABCC3 gene can confer susceptibility to IBD, the mechanism underlying this remains tentatively unknown. Recent data have shown that bile acids can play a role in gut barrier

defence and the maintenance of luminal microflora. Classically, the dominant role of bile acids is to aid the absorption of lipid absorption by micellar solubilization. A second novel role, that is the inhibition of bacteria growth in the distal small bowel and colon, has been now been suggested and supported by several lines of evidence. In liver cirrhosis in both humans (Bauer et al. 2002) and animals(Lorenzo-Zuniga et al. 2006), decreased bile acid secretion leads to secondary bacterial overgrowth occurs ; and in animals, the ligation of bile duct leads to similar picture (Slocum et al. 1992; Ding et al. 1993) ; bile and unconjugated bile acids have also been shown to inhibit bacterial growth *in vitro*(Binder, Filburn, and Floch 1975; Begley, Gahan, and Hill 2005). By feeding of bile or conjugated bile acids in conditions of bile acid deficiency in the intestine, a reduction/abolition of bacterial overgrowth and bacterial translocation to intestinal lymph nodes have been demonstrated(Lorenzo-Zuniga et al. 2006; Ding et al. 1993). In a seminal study, Inagaki and colleagues presented further compelling evidence, based on mice studies, that the antibacterial effect of conjugated bile acid in the distal intestine is mediated by a cellular pathway involving a further nuclear receptor, the farnesoid X receptor (FXR), an orphan receptor that is activated by conjugated bile acids(Inagaki et al. 2006). Genetic variations of a further transcriptional regulator of bile acid transporters (including MRP3), Steroid and Xenobiotic receptor (SXR/PXR), have been shown to influence survival and modify disease progression in primary sclerosing cholangitis, which is extricably associated with IBD(Karlsen T.H. et al. 2006).

We therefore postulate that defective bile acid metabolism mediated by inherited variations of the ABCC3 gene may confer susceptibility to IBD (by virtue of reducecd mucosal protection). In our current analyses, the existing interaction between

PXR/NR1I2 and *ABCC3* is pertinent and points to the importance of the xenobiotic-metabolism pathway in the pathogenesis of IBD. Emerging data show that the xenobiotics response/defence mediated via several key transcriptional regulators such as Pregnane-X receptor, are also intricately linked to NFKB signalling pathways and inflammatory response(Zhou et al. 2006b; Gu et al. 2006a). These again highlight the multifactorial model of IBD pathogenesis; and in the case of *ABCC3 /MRP3* may influence susceptibility critically in the presence of other genetic and environmental factors. Our current data further supports the importance of xenobiotic-and efflux-transporters and suggest a novel role in ABCC3/MRP3 gene and bile acid transport in determining IBD-susceptibility.

It is noteworthy that 4 out 8 tSNPs used demonstrated significant associations with IBD in the Scottish dataset. In contrast, the negative findings on a single locus level as evident in the Swedish dataset did not exclude the involvement of this gene on a haplotypic level. Several explanations are possible: Firstly, the current statistical approach used in this study focused on the coefficient of determination (i.e. haplotype r^2) in a regression model uses haplotypes defined by the tSNPs to predict the state of the tagged SNPs. This method was employed as it offered considerable economy and power over single marker approaches(de Bakker et al. 2005). The current data suggests that the collective 8-locus haplotype markers are capturing an unknown variant for which the singular tSNPs are not in the Swedish cohort. The apparent differences between single locus and multimarker haplotypic associations reflect the fundamental differences between statistical power, efficiency and analyses. Further examples of highly significant 'haplotypic' associations observed in parallel with marginal 'genotypic' associations have also been well-demonstrated in complex

disease, in the examples of *NOD1* gene sliding haplotypes with IBD(McGovern et al. 2005); and the *ADAM33* gene with asthma(Van Eerdewegh et al. 2002). Secondly, the smaller sample size in the Swedish cohort may diminish the power to replicate single variant association significantly. However, by comparing the commonly inferred haplotypes in IBD and controls between these two populations, we have not detected significant differences in between respective Scottish and Swedish case/control groups. This suggests that despite obvious phenotypic heterogeneity between Scottish and Swedish datasets, the argument for genetic heterogeneity is minimised as demonstrated in recent studies in NOD2/CARD15 frequencies between these populations (Arnott et al. 2004a; Torkvist et al. 2006a).

In conclusion, our present study provides compelling evidence for the involvement of the ABCC3/MRP3 gene in determining susceptibility to IBD. This and recent genetic studies highlight the critical importance of barrier function in the aetiopathogenesis of IBD. Elucidating the functional mechanisms of ABCC3/MRP3 together with ABCB1/MDR1 and their respective crosstalk with the innate immunity will provide new insights into shared causal mechanisms underlying intestinal inflammatory diseases.

Table 1a: Demographics and clinical characteristics of UC study population (n=387). Disease location (E1 – proctitis, E2 – left sided disease and E3 – extensive colitis) and severity (S3- severe UC as defined by Truelove and Witts criteria) are defined using the Montreal Classification(Silverberg et al. 2005). †-Unknown status not used in the description of smoking status and disease extent. ‡Surgery rates in cohort with full phenotypic data only (n=250).

Characteristics	**Scottish UC (n=387)**	**Swedish UC (n=284)**
Sex (M/F)	209/178	163/121
Age of onset (years)	35.2 (25.9-50.4)	28.4 (20.4-41.5)
Age of onset <16 years	11 (2.8%)	27 (9.5%)
Current smoker	43 (11.1%)	22 (9.9%)
Ex-smoker	154 (39.8%)	109 (49.1%)
Never smoked	188 (48.6%)	90 (40.5%)
Unknown	2 (0.55)	72†
Disease extent		
E1	70 (18.1%)	25 (10.0%)
E2	165 (42.6%)	110 (44.0%)
E3	136 (35.1%)	115 (46.0%)
Unknown	16 (4.1%)	34†
Disease severity		
S3	124 (32.0%)	-
Surgery for S3	79 (30.4%)	39 (15.6%)‡
Extra-intestinal manifestations	45 (11.6%)	-
Primary Sclerosing Cholangitis	7 (1.8%)	12 (4.8%)
Drug therapy		
Azathioprine	107 (27.6%)	-

Table 1b: Demographics and clinical characteristics of patients with CD. Disease location (L1-Ileal, L2-colonic, L3-ileocolonic, L4-upper GI); behaviour (B1-inflammatory, B2-stricturing, B3-penetrating and p-perianal) and age of onset (A1-<16 years, A2- 17-40 years and A3->40 years) were defined according to the Montreal Classification.

Characteristics	Scottish Crohn's disease (n=328)	Swedish Crohn's disease (n=192)
Sex (M/F)	120/208	109/108
Smoking		
Current	88 (26.8%)	39 (23.5%)
Ex-smoker	136 (41.5%)	62 (37.4%)
Never	93 (28.4%)	64 (38.6%)
Unknown	11 (3.3%)	
Age of onset, years	28.5	24.8
Median (+IQR)	(21.0-41.1)	(18.7-37.2)
Age of diagnosis		
A1	28 (8.5%)	34 (15.7%)
A2	215 (65.6%)	135 (62.2%)
A3	85 (25.9%)	48 (22.1%)
Drug therapy		
Infliximab	45 (13.7%)	-
Azathioprine	135 (41.2%)	-
Disease Location		
L1	108 (33.0%)	29 (13.4%)
L1+L4	7 (2.2%)	6 (2.8%)
L2	108 (33.0%)	103 (47.7%)
L2+L4	4 (1.2%)	0 (0%)
L3	77 (20.4%)	66 (30.6%)
L3+L4	15 (4.6%)	2 (0.93%)
L4	13 (4.0%)	0 (0%)
Unknown	5 (1.5%)	9 (4.17%)
Disease behaviour		
B1	171 (52.1%)	139 (69.5%)
B1p	40 (12.2%)	3 (1.5%)
B2	41 (12.5%)	39 (19.5%)
B2p	4 (1.2%)	2 (1.0%)
B3	54 (16.5%)	7 (3.5%)
B3p	9 (2.7%)	9 (4.5%)
Unknown	9 (2.7%)	17
Surgery	216 (65.8%)	102 (47.0%)
Extra-intestinal manifestations	118 (43.1%)	-
PSC		4 (1.8%)

Table 2: Selected tagging SNPs for ABCC3.

Gene (tSNP reference sequence nos.)	Legend	
rs757421	tSNP1	Upstream/Intergenic
rs2412333	tSNP2	Intronic
rs739921	tSNP3	Intronic
rs12051822	tSNP4	Intronic
rs2301837	tSNP5	Intronic
rs879459	tSNP6	Intronic
rs2277624	tSNP7	Synonymous Coding
rs3785911	tSNP8	Intronic/Downstream

Table 3: Association analyses of ABCC3 SNPs in inflammatory bowel disease

	Inflammatory bowel disease			Controls			
	Allele counts			Allele counts			
	Major	Minor	MAF	Major	Minor	MAF	P-value by cohort
tSNP1							
Scottish (714/326)	969	459	0.32	474	178	0.27	**0.03** (1.26, 1.02-1.54)
Swedish (461/267)	635	287	0.31	375	157	0.29	0.55 (1.08, 0.85-1.36
tSNP2							
Scottish (705/337)	1043	367	0.26	486	188	0.28	0.39 (0.91, 0.74-1.12)
Swedish (476/267)	753	199	0.28	426	108	0.20	0.81 (1.04, 0.80-1.36)
tSNP3							
Scottish (717/338)	1013	421	0.29	512	164	0.24	**0.016** (1.30,1.05-1.60)
Swedish (465/261)	644	284	0.31	368	154	0.30	0.70 (1.05,0.83-1.33)
tSNP4							
Scottish (661/336)	1013	309	0.23	497	175	0.26	0.21 (0.87, 0.69-1.07)
Swedish (466/268)	731	201	0.22	423	113	0.21	0.88 (1.03, 0.79-1.33)
tSNP5							
Scottish (697/328)	1302	122	0.09	589	67	0.10	0.26 (0.82, 0.60-1.13)
Swedish (476/275)	891	61	0.06	503	39	0.07	0.63 (0.88, 0.58-1.34)
tSNP6							
Scottish (711/338)	1218	204	0.14	609	67	0.10	**0.0058** (1.52, 1.14-2.04)
Swedish (468/264)	748	188	0.20	427	101	0.19	0.71 (1.06, 0.81-1.39)

tSNP7							
Scottish (620/327)	964	276	0.22	544	110	0.17	**0.0063** (1.42, 1.11-1.81)
Swedish (468/255)	704	232	0.25	387	123	0.24	0.83 (1.04, 0.81-1.33)
tSNP8							
Scottish (699/337)	1025	373	0.27	468	206	0.31	0.07 (0.83, 0.67-1.01)
Swedish (475/270)	651	299	0.31	376	164	0.30	0.70 (1.05, 0.84-1.32)

Table 4: Association analysis of ABCC3 SNPs in Crohn's disease and ulcerative colitis sub-phenotypes

	Crohn's disease			Ulcerative Colitis			Healthy Controls			P-value CD	P-value UC
	Allele Counts			Allele Counts			Allele Counts				
	Major	Minor	MAF	Major	Minor	MAF	Major	Minor	MAF		
tSNP1											
Scottish	432	226	0.343	537	233	0.303	474	178	0.273	**0.006** (1.40,1.10-1.70)	0.24 (1.15, 0.92-1.46)
Swedish	266	106	0.285	369	181	0.329	375	157	0.295	0.77 (0.95, 0.71-1.28)	0.24 (1.17, 0.91-1.52)
tSNP2											
Scottish	507	145	0.222	536	222	0.293	486	188	0.279	**0.01** (0.73, 0.58-0.94)	0.80 (1.07, 0.85-1.35)
Swedish	299	85	0.221	454	114	0.201	426	108	0.202	0.51 (1.12, 0.81-1.55)	1.00 (0.99, 0.74-1.33)
tSNP3											
Scottish	463	209	0.311	550	212	0.278	512	164	0.242	**0.005** (1.41, 1.11-1.78)	0.13 (1.20, 0.95-1.52)
Swedish	248	114	0.315	396	170	0.303	368	154	0.295	0.55 (1.10, 0.82-1.47)	0.89 (1.03, 0.79-1.33)
tSNP4											
Scottish	506	144	0.222	507	165	0.245	497	175	0.260	0.11 (0.81, 0.63-1.04)	0.57 (0.92, 0.72-1.18)
Swedish	300	78	0.206	431	123	0.222	423	113	0.211	0.93 (0.97, 0.70-1.35)	0.66 (1.07, 0.80-1.43)
tSNP5											
Scottish	567	63	0.10	705	59	0.08	589	67	0.102	0.93 (1.02, 0.71-1.74)	0.11 (1.36, 0.94-1.96)
Swedish	359	25	0.07	532	36	0.06	503	39	0.07	0.79 (0.90, 0.53-1.51)	0.63 (0.87, 0.55-1.39)
tSNP6											
Scottish	574	82	0.125	644	122	0.159	609	67	0.010	0.14 (0.77, 0.55-1.08)	**0.008** (1.72, 1.25-2.38)
Swedish	314	70	0.182	434	118	0.214	427	101	0.191	0.80 (0.94, 0.67-1.32)	0.36 (1.15, 0.85-1.55)

tSNP7											
Scottish	479	127	0.210	485	149	0.235	544	110	0.168	0.06 (1.31, 0.99-1.75)	**0.003** (1.51, 1.15-2.00)
Swedish	279	93	0.246	425	139	0.250	387	123	0.241	0.85 (1.12, 0.55-2.25)	0.81 (1.05, 0.77-1.43)
tSNP8											
Scottish	491	157	0.242	534	216	0.288	468	206	0.305	**0.01** (1.38, 1.08-1.76)	0.49 (1.09, 0.87-1.52)
Swedish	256	128	0.333	395	171	0.302	376	164	0.304	0.35 (1.15, 0.87-1.52)	1.00 (0.99, 0.77-1.28)

Table 5a: Log-likelihood significance between the common haplotypes of ABCC3 gene haplotypes and IBD/UC/CD. Significance for association is calculated using the test statistic *2*(ln(L case)+ln(Lcontrol)-ln(Lcase/Lcontrol)*, which has a χ^2 distribution with n-1 degrees of freedom (where n=number of inferred haplotypes)

	Log-likelihood P-value	
Phenotype	**Scottish**	**Swedish**
IBD	**0.00046**	**0.001**
UC	**0.004**	**0.003**
CD	**0.001**	**0.004**
Current smoker IBD	0.29	0.32
ABCB1/MDR1 G-allele (rs3789243)	0.79	-
PXR/NR1I2 C-allele (rs1523127)	**0.005**	-
CD		
Current smoker	0.11	0.21
A1	0.19	0.12
A2	0.08	0.09
A3	0.50	0.25
L1 + (L1+L4)	0.19	0.65
L1	0.15	0.69
L2+ (L2+L4)	0.35	0.07
L2	0.28	0.08
L3+ (L3+L4)	**0.02**	**0.03**
L3	**0.02**	**0.03**
L4	0.92	0.89
B1 + B1p	**0.005**	**0.01**
B1	**0.006**	**0.01**
B2 + B2p	0.84	0.78
B2	0.71	0.64
B3+ B3p	0.62	0.81
B3	0.59	0.79
UC		
E1	0.46	0.71
E2	0.49	0.42
E3	**0.04**	0.08
S3	0.89	0.13
Surgery	0.81	0.48

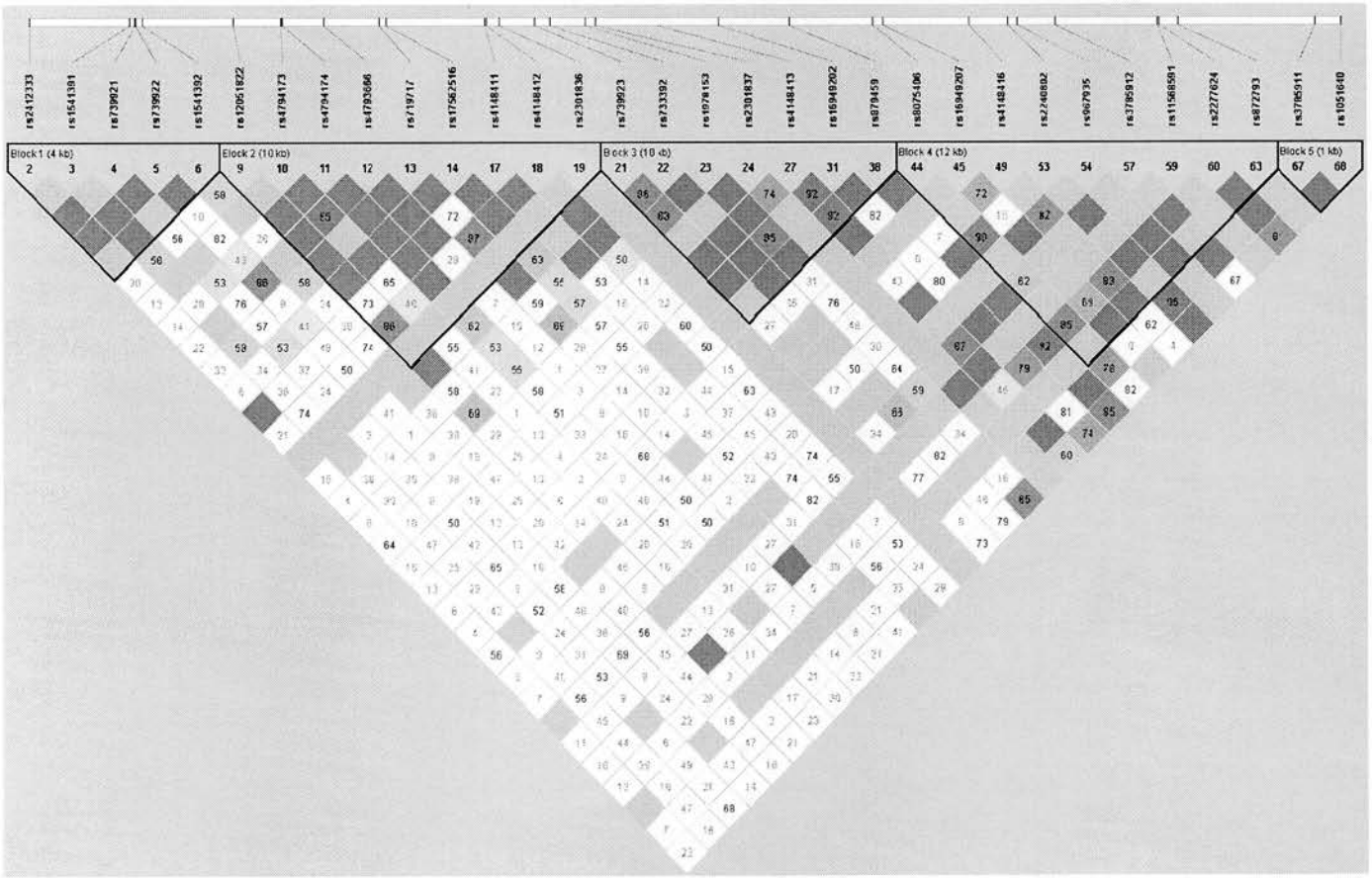
Table 5b: Stratified log-likelihood analyses based on previously implicated A-allele of the PXR rs1523127 SNP (Dring et al.) using 8 tSNP set.

rs1523127/-24381	**A**	**C**
IBD (n=715)	858 (60.0%)	572 (40.0%)
HC (n=334)	405 (60.6%)	263 (39.4%)
Log-likelihood analysis on stratified sets based on A/C allele carriage	0.005	0.06

Table 6: Comparisons of ABCC3 estimated haplotype frequencies in IBD, UC and CD populations and healthy controls. *-Significantly higher no. of haplotypes for CD population for this haplotype, p= 0.02, odds ratio 1.76, 95% CI 1.11-2.

		Scottish cohort				Swedish			
	Haplotype	**IBD (%)**	**UC (%)**	**CD (%)**	**HC (%)**	**IBD (%)**	**UC (%)**	**CD (%)**	**HC (%)**
1.	22221222	13.3	11.6	15.4	12.8	11.3	10.9	11.7	12.7
2.	22121222	5.5	4.5	**6.8***	4.0	4.9	4.7	5.2	5.4
3.	12221222	4.8	4.5	5.1	5.7	5.0	5.5	4.4	5.2
4.	22221221	4.8	4.5	5.1	5.7	5.1	4.5	5.8	5.5
5.	21221222	4.6	4.9	4.2	5.0	3.1	2.9	3.4	3.2
6.	22221212	3.9	3.7	4.0	2.7	3.8	3.7	4.0	3.9
7.	22211222	3.8	3.6	4.1	4.6	3.2	3.2	3.1	3.4
8.	22221122	2.3	2.4	2.1	1.5	2.9	3.1	2.6	2.8
9.	12121222	2.0	1.7	2.2	1.8	2.2	2.4	2.0	2.2
10.	22121221	2.0	1.7	2.2	1.8	2.2	1.9	2.6	2.4

Figure 1: Linkage disequilibrium of ABCC3 gene using HapMap CEU data (HapMap data release 21, July 2006 National Centre for Biotechnology Information [NCBI] build 35 assembly, dbSNP build 125). Current selected tSNPs encircled and represented in each haplotype block (using the solid spine criteria in the Haploview programme).



Chapter 9

Implications

Implications

The current data generated from this thesis have several key and novel implications. Firstly, the importance and predictability of corticosteroid resistance and dependence in inflammatory bowel disease have now been demonstrated. The inception cohort in this study, represents the most recent observation on the natural history of inflammatory bowel disease and corticosteroid usage (compared with population-based cohorts in 1970-80s). This study also highlighted the key feature in the variability of the clinical course of IBD is determined by the response to corticosteroids in particular, in patients with extensive UC at time of diagnosis and the phenotype of fistulising disease in Crohn's. It is pertinent that with the safety issues surrounding the new biological therapies (such as the developments of hepatosplenic T-cell lymphoma and progressive multifocal leukoencephalopathy associated with infliximab and natalizumab respectively) becoming increasingly relevant in clinical practice, there remains a need to understand the mechanisms underlying corticosteroid resistance/dependence and thereby, allowing appropriate stratification of patients to more potent therapies (reducing both biological and corticosteroid-related toxicity)(Mackey et al. 2007; Van Assche et al. 2005). Secondly, the development of a robust predictive model (the Ho-index) which stratifies patient according to the risk of non-response to intravenous corticosteroids in acute severe UC, also represents further progress in the above mentioned strategy of targeting therapy towards subsets of patients who are most likely to achieve maximal benefit. The current model was developed using the largest series of acute severe UC, and has its advantages in identifying subsets of patients at low-, intermediate and high-risk of failed medical therapy. This is again, particularly relevant in the context of risk-

counselling. This study propose the use of second-line medical therapies such as infliximab or ciclosporin in patients in the intermediate risk group (risk score 2-3). These clincial studies have critically provided the assimilation of highly accurate phenotypic and follow-up data of these patients, permitting very detailed statistical analyses to be performed.

Thirdly, the identification of the ABCB1/MDR1 and ABCC3/MRP3 genes as susceptibility genes in UC (and IBD for the latter) further implicate the importance of gut barrier defence and xenobiotic-metabolizing pathways in the aetiopathogenesis of IBD. In addition to animal data (mdr1a-knock out colitis model for example), functional studies have now demonstrated key signalling cross-talk between Pregnane X-receptor gene (transcriptional regulator of ABCB1/MDR1 and ABCC3/MRP3), with NFKB and inflammatory response(Gu et al. 2006b; Zhou et al. 2006a). It is of interest to note that variations of both genes are associated primarily with extensive disease – again highlighting a further critical factor in future association studies, namely phenotypic heterogeneity. Fourthly, I have now demonstrated the feasibility and applicability of the gene-wide haplotype approach in association studies in complex diseases, in its use in ABCB1/MDR1 gene (Chapter 6). This represented one of the earliest study employing the current technique which is now a widely accepted approach with the availability of dense maps of the genome via publicly available database such as the HapMap Project and ENCODE.

Future directions

The implications as discussed in the earlier section, logically follow on to further important questions :

- Given the predictability of corticosteroid resistance/dependence in IBD, the potential pharmacogenomic contribution to this phenomenon should be investigated. Although the molecular mechanisms of corticosteroids in inflammation are well-known, the innate variability of response is ill-understood. Therefore, the next logical step will be to develop a comprehensive pharmacogenetic model in corticosteroid resistance using IBD as the inflammatory disease model. I have now employed a high-throughput analysis involving 70 (768 tSNPs) key genes in corticosteroid-related pathways and epithelial drug transport proteins using both 1-year inception outcomes and also a novel Cox-regressional hazard model in our detailed IBD cohort. This currently funded study is unique in its longitudinal approach and the genetic coverage of candidate genes. I intend to rapidly extend this study to inter-population studies (including paediatric IBD cohorts) and also other inflammatory diseases (pertinently – asthma) (Translational Reseach Grant, Chief Scientist Office – Dr. Gwo-Tzer Ho 2006).

- In acute severe UC, I have demonstrated that clinical markers of disease severity can be assimilated into statistical models (Ho-index) with good performance in sensitivity/specificity to predict the outcome of an acute attack of severe UC. It is noteworthy that other important predictive factors of clinical course such as the

propensity to develop severe disease and the identification of earlier markers of poor outcome remain elusive. Presently, genetic variants associated with extensive and severe disease, at best, only modestly predict medically resistant UC or surgery. There remains a need to identify early biomarkers of severe disease and outcome. I have considered two further rational approaches : Firstly, the use of proteomics both in sera and faeces, in identifying early biomarkers of corticosteroid resistance/surgery are funded and in progress. (Project Grant, Chief Scientist Office 2006). Secondly, the use of genome wide association studies (GWAS) to further investigate genetic variants of disease severity and extent are in progress, and the results are eagerly awaited. Both complementary approaches promise to provide new insights both to disease outcome and pathogenesis. Furthermore, I have now completed a recent pilot study in the use of faecal calprotectin to predict outcome in acute severe UC.

- The functional consequence of the implicated sequence variations of ABCB1/MDR1 and ABCC3/MRP3 will provide critical new insights to disease pathogenesis. I have now fully characterised the expression patterns of the entire ABC-transporter superfamily (Ho G.T., et al. 2007). The most striking observation again, is the downregulation of MDR1 in UC. MDR1 expression is significantly lower in UC (basal and inflamed states; $Fold_{change}$ 1.9 and 1.8; p= $9.0x10^{-10}$ and $5.6x10^{-6}$ compared with inflamed/non-inflamed controls respectively). MDR1 constitutive expression demonstrates a decreasing gradient distally, lowest in the rectosigmoid region (p=0.001 left vs. right colonic

expression). (with a expression gradient – lowest in rectosigmoid region). In addition to this, a further 15 (31%) genes were differentially expressed in IBD. Functional work is currently focussed in elucidating the exact mechanism of ABCB1/MDR1 and ABCC3/MRP3 in the maintenance of gut barrier defence in inflammatory bowel disease. For both these genes, the current working hypothesis is that low MDR1 and MRP3 expressions predispose to inflammation and high levels protect. The modulations of the expressions of these proteins at the epithelial level and therein determine susceptibility to disease can be evaluated. The utilisation of T_{H2} colitis model (Oxazolone) and transgenic B27 rat colitis models with the modulation of MDR1/MRP3 expressions (either via PXR and other known environmental inducers to upregulate ; and known inhibitors or silencing RNAs to downregulate) with the assessement of the consequent severity of colitis is proposed. In addition to this, the mechanisms can be further explored using an in vivo model to assess the exact mechanistic effects of MDR1 function (for example) in response to exposure to pathogen-associated protein motifs (such as lipopolysaccharide, muramyl dipeptide and flagellin) and its crosstalk with other aspects of innate immunity such as toll-like receptors, inflammatory signalling pathways and regulatory proteins. By establishing the co-regulatory signalling pathways, the specific effects of the implicated variants of ABCB1/MDR1 and ABCC3/MRP3 can also be investigated (*Clinican Scientist Fellowship grant in preparation*).

Chapter 10

Appendix

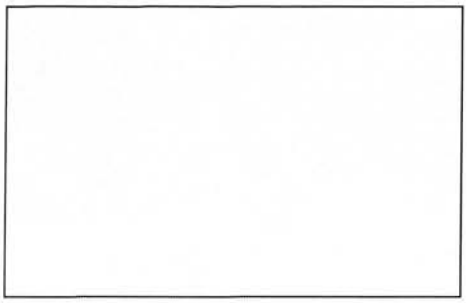
Proforma datasheet

Example shown below:

The clinical course of severe ulcerative colitis

Date of admission:

Date of diagnosis:



Outcome of severe UC

Colectomy ☐ _____ (date)

Day 7 criteria		**Discharge criteria**	
Partial response	☐	Partial response	☐
Complete response	☐	Complete response	☐

Disease extent

At diagnosis_____		At latest follow-up_____	
Proctitis	☐	Proctitis	☐
Left-sided (<splenic)	☐	Left-sided (<splenic)	☐
Extensive (splenic)	☐	Extensive (splenic)	☐

Details of severe UC

Truelove and Witts? ☐

Stool >6 on admission ☐

Fever >37.8C ☐

Mean pulse >90 ☐

ESR >30 ☐

HB <10.5 ☐

Severity of presentation (during 1st 3 days of i.v. steroid therapy)

Stool f1	
Stool f2	
Stool f3	
Albumin 1-3	
CRP 1-3	
ESR 1-3	
Hb 1-3	
WCC 1-3	
Presence of fever 1-3	
Colonic dilatation 1-3	

These are indices taken from 1st 3 days of GCS therapy. For blood results put in any results in chronological order within the 1st 3 days of therapy.

Details of patient

Previous presentations or hospitalization? ☐

Drugs on admission:

5-ASA: YES / NO
Which type: Asacol / Pentasa / Balsalazide / Sulphasalsazine / Osalazine

Oral Prednisolone YES / NO

Drugs therapy for severe UC :

IV HC or 6MP (circle): YES / NO

How long for? :_____(days)

IV cyclosporine : YES / NO

Date started :_____(days)

How long for? :_____(days)

Drugs on discharge :

Azathioprine started? YES / NO

_____ (date started)

Other details:

Before discharge

Date of discharge: _____

C-reactive protein	
Stool frequency 24 hours prior to discharge	
Albumin	
ESR (if available)	

Subsequent course

Date of latest follow-up:_____

Relapse: YES / NO

Time to relapse	Date	Therapy
1st		
2nd		
3rd		
4th		
5th		

Surgery: YES / NO

Date of surgery: _____

Need for further immunosuppression: YES / NO

Date of commencement: _____

Corticosteroid Pharmacogenetics Study

Section 1. (circle relevant options)	
DISEASE: UC / CD **MANAGED AS:** In-Patient / Out-Patient	
Date of Diagnosis (more or less when patient was officially diagnosed or when IBD treatment implemented):	Date of Latest Follow-up: Date of Case Notes Review:

Patient Details:
NAME:
Gender:
Date of Birth:
Hospital No.:

Smoking Status
Active / Ex-smoker / Non-smoker

Section 2(a). Drug Therapy At Diagnosis/Index Admission (*circle relevant drugs options*)			
TOPICAL STEROID: Foam / Suppository 5-ASA: Foam / Suppository	5-ASA (ORAL) Pentasa / Asacol / Salazopyrin/ Sulphasalazine / Balsalazide Dose:	OTHERS (ORAL) Infliximab / Ciclosporin / Azathioprine / 6MP / Diet Therapy Dose:	CORTICOSTEROIDS IV / ORAL Dose: Date Started: Date Ended (optional): Duration (optional):

Section 2 (b). Corticosteroid (CS) Commencement (fill in if CS-therapy was NOT started at Diagnosis/Index Admission)				
IV / ORAL	Date Started	Date Ended	Duration	Dose

Section 3. Immediate Outcome at 30 days (DATE: _____) after Corticosteroid

Commencement *(refer to Section 2(a) or 2(b) for Start Date of CS-therapy)*

NO RESPONSE	PARTIAL REMISSION	COMPLETE REMISSION
No regression, and requiring: • Surgery • Infliximab • Cyclosporin • Azathioprine / 6MP/ Methotrexate • ORAL steroids step-up to IV	Regression of symptoms, e.g: • BO ≤ 4 /day • Stools: No blood/pus/mucus • No abdominal pain • OR All above symptoms, but not present daily AND no fever, weight loss, extra-intestinal symptoms	Total regression of symptoms: • BO ≤ 2 /day • Stools: No blood/pus/mucus • No abdominal pain, fever, weight loss, extra-intestinal symptoms
STEROID DOSE at 30 DAYS (optional):		
OTHER DETAILS		

Section 4: Intermediate Outcome at 90 days (DATE: _____) after Corticosteroid Commencement *(refer to Section 2(a) or 2(b) for Start Date of CS-therapy)*

No Response	CS-Dependent	Complete Remission
No regression; requiring either: • Surgery • Infliximab • Cyclosporin • Azathioprine / 6MP / Methotrexate • ORAL step-up to IV Steroids	Either • Failure to wean, i.e recurrence of active disease upon dose reduction • Flare within 30 days of complete CS withdrawal • Steroid Courses: ≥ 2 within 90 days • Addition/Escalation of other therapies	Either • Off steroids, without need for further treatment
STEROID DOSE at 90 DAYS (optional):		
OTHER DETAILS:		

Section 5: OUTCOME at 1 YEAR (DATE: _____) after Corticosteroid Commencement *(refer to Section 2(a) or 2(b) for Start Date of CS-therapy)*

No Response	CS-Dependent:	Prolonged Remission
No regression; requiring either: • Surgery • Infliximab / Azathioprine	Either • Failure to wean • Flare within 30 days of CS withdrawal • Steroid Courses: ≥ 3 within 1yr	Either • ≥ 30 days steroid-free remission • Steroid Courses: ≤ 2 within 1yr

	• Azathioprine / 6MP / Methotrexate • Infliximab / Other TNF-therapies	

STEROID DOSE at 1 YEAR (optional)
OTHER DETAILS:

Notes:

1. Disease severity/location/extent should already be in database.
2. All patients with corticosteroid therapy should fall into each of the categories stated.
3. Upon initial diagnosis, we shall include data of all corticosteroid therapy within 3 months of diagnosis: this is to capture all treatments (e.g. patients who had been started on milder treatments such as 5ASA for example).
4. Please note subheadings for major outcome points at 3, 6 and 12 months.

For UC:

Disease extent: E1 (proctitis)
E2 (left sided – up to splenic flexure)
E3 (extensive – beyond splenic flexure)

Disease severity:
S3 (fulfilling modified Truelove and Witts criteria): "≥ 6 episodes of bloody diarrhoea/24 h with one or more of the following features: Anaemia (<10.5 g/dl); Fever (>37.8 °C); Tachycardia (>90/min); ESR >30 mm/h)

For CD:

Disease location: Ileal (L1)
Colonic (L2)
Ileo-colonic (L3)
Upper GI (L4)

Disease behaviour: Inflammatory (B1)
Stricturing (B2)
Penetrating (B3)
(Add p – if penetrating)

References

1. (2005) A haplotype map of the human genome. *Nature* 437 (7063):1299-1320.

2. (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447 (7145):661-678.

3. Achkar JP et al (2006) Phenotype-stratified genetic linkage study demonstrates that IBD2 is an extensive ulcerative colitis locus. *Am.J.Gastroenterol.* 101 (3):572-580.

4. Actis GC et al (1998) Efficacy and efficiency of oral microemulsion cyclosporin versus intravenous and soft gelatin capsule cyclosporin in the treatment of severe steroid-refractory ulcerative colitis: an open-label retrospective trial. *Inflamm.Bowel.Dis.* 4 (4):276-279.

5. Adcock IM et al (1995) Abnormal glucocorticoid receptor-activator protein 1 interaction in steroid-resistant asthma. *J.Exp.Med.* 182 (6):1951-1958.

6. Ahmad T et al (2002) The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 122:854-866.

7. Ahmadi KR et al (2005) A single-nucleotide polymorphism tagging set for human drug metabolism and transport. *Nat.Genet.* 37 (1):84-89.

8. Aithal GP et al (2001) Role of polymorphisms in the interleukin-10 gene in determining disease susceptibility and phenotype in inflamatory bowel disease. *Dig Dis Sci* 46 (7):1520-1525.

9. Almer S et al (1995) Plain X-ray films and air enema films reflect severe mucosal inflammation in acute ulcerative colitis. *Digestion* 56 (6):528-533.

10. Altshuler D et al (2000) The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat.Genet.* 26 (1):76-80.

11. Ang YS et al (2000) Randomized comparison of unfractionated heparin with corticosteroids in severe active inflammatory bowel disease. *Aliment Pharmacol Ther* 14 (8):1015-1022.

12. Ansari A et al (2002) Thiopurine methyltransferase activity and the use of azathioprine in inflammatory bowel disease. *Aliment Pharmacol Ther*16 (10):1743 -1750.

13. Ardizzone S et al (2006) Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. *Gut* 55 (1):47-53.

14. Armuzzi A et al (2003) Genotype-phenotype analysis of the Crohn's disease susceptibility haplotype on chromosome 5q31. *Gut* 52 (8):1133-1139.

15. Arnott ID et al (2004a) NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? *Genes Immun.* 5 (5):417-425.

16. Arnott ID et al (2004b) NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? *Genes Immun.* 5 (5):417-425.

17. Asghar A et al (2002) Induction of multidrug resistance-1 and cytochrome P450 mRNAs in human mononuclear cells by rifampin. *Drug Metab Dispos* 30 (1):20-26.

18. Askling J et al (2001) Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 120 (6):1356-1362.

19. Ayres RC et al (1996) Progression of ulcerative proctosigmoiditis: incidence and factors influencing progression. *Eur J Gastroenterol Hepatol* 8 (6):555-558.

20. Bach SP and Mortensen NJ (2006) Revolution and evolution: 30 years of ileoanal pouch surgery. *Inflamm.Bowel.Dis.* 12 (2):131-145.

21. Back DJ and Rogers SM (1987) Review: first-pass metabolism by the gastrointestinal mucosa. *Aliment.Pharmacol.Ther.* 1 (5):339-357.

22. Barnes PJ (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin.Sci.(Lond)* 94 (6):557-572.

23. Barnes PJ (2004) Corticosteroid resistance in airway disease. *Proc.Am.Thorac.Soc.* 1 (3):264-268.

24. Bauer TM et al (2002) Small intestinal bacterial overgrowth in human cirrhosis is associated with systemic endotoxemia. *Am.J.Gastroenterol.* 97 (9):2364-2370.

25. Baumgart DC et al (2006) Tacrolimus is safe and effective in patients with severe steroid-refractory or steroid-dependent inflammatory bowel disease--a long-term follow-up. *Am.J Gastroenterol* 101 (5):1048-1056.

26. Beaugerie L et al (2001) Impact of cessation of smoking on the course of ulcerative colitis. *Am.J Gastroenterol* 96 (7):2113-2116.

27. Begley M, Gahan CG, and Hill C (2005) The interaction between bacteria and bile. *FEMS Microbiol.Rev.* 29 (4):625-651.

28. Benet LZ and Cummins CL (2001) The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* 50 Suppl 1:S3-11.

29. Bibiloni R et al (2005) VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am.J Gastroenterol* 100 (7):1539-1546.

30. Binder HJ, Filburn B, and Floch M (1975) Bile acid inhibition of intestinal anaerobic organisms. *Am.J.Clin.Nutr.* 28 (2):119-125.

31. Black AJ et al (1998) Thiopurine methyltransferase genotype predicts therapy-limiting severe toxicity from azathioprine. *Ann Intern Med* 129 (9):716-718.

32. Boirivant M et al (1996) Stimulated human lamina propria T cells manifest enhanced Fas-mediated apoptosis. *J Clin.Invest* 98 (11):2616-2622.

33. Bouma G et al (1999) Genetic markers in clinically well defined patients with ulcerative colitis (UC). *Clin Exp Immunol* 115 (2):294-300.

34. Bouma G et al (1996a) Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease. *Scand J Immunol* 43 (4):456-463.

35. Bouma G et al (1996b) Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol* 103 (3):391-396.

36. Brant SR et al (2003) MDR1 Ala893 polymorphism is associated with inflammatory bowel disease. *Am.J.Hum.Genet.* 73 (6):1282-1292.

37. Buckell NA and Lennard-Jones JE (1979) How district hospitals see acute colitis. *Lancet JID - 2985213R* 1 (8128):1226-1229.

38. Buckell NA et al (1979) Measurement of serum proteins during attacks of ulcerative colitis as a guide to patient management. *Gut* 20 (1):22-27.

39. Burich A et al (2001) Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 281 (3):G764-G778.

40. Burkett KM et al (2005) A comparison of five methods for selecting tagging single-nucleotide polymorphisms. *BMC.Genet.* 6 Suppl 1:S71.

41. Cahill RJ et al (1997) Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with Helicobacter hepaticus. *Infect Immun* 65 (8):3126-3131.

42. Campbell S, Travis S, and Jewell D (2005) Ciclosporin use in acute ulcerative colitis: a long-term experience. *Eur J Gastroenterol Hepatol* 17 (1):79-84.

43. Caprilli R et al (1987) Early recognition of toxic megacolon. *J.Clin.Gastroenterol.* 9 (2):160-164.

44. Carbonnel F et al (1996) Intravenous cyclosporine in attacks of ulcerative colitis: short-term and long-term responses. *Dig Dis Sci* 41 (12):2471-2476.

45. Carbonnel F et al (2000) Predictive factors of outcome of intensive intravenous treatment for attacks of ulcerative colitis. *Aliment Pharmacol Ther* 14 (3):273-279.

46. Carmichael J et al (1981) Corticosteroid resistance in chronic asthma. *Br.Med.J.(Clin.Res.Ed)* 282 (6274):1419-1422.

47. Carter MJ et al (2001a) The interleukin 1 receptor antagonist gene allele 2 as a predictor of pouchitis following colectomy and IPAA in ulcerative colitis. *Gastroenterology* 121 (4):805-811.

48. Carter MJ et al (2001b) Association of the interleukin 1 receptor antagonist gene with ulcerative colitis in Northern European Caucasians. *Gut* 48 (4):461-467.

49. Carter MJ, Lobo AJ, and Travis SP (2004) Guidelines for the management of inflammatory bowel disease in adults. *Gut* 53 Suppl 5:V1-16.

50. Cascorbi I et al (2001b) Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. *Clin Pharmacol Ther* 69 (3):169-174.

51. Cascorbi I et al (2001a) Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. *Clin Pharmacol Ther* 69 (3):169-174.

52. Chakravarty BJ (1993) Predictors and the rate of medical treatment failure in ulcerative colitis. *Am J Gastroenterol* 88 (6):852-855.

53. Chapman JM et al (2003) Detecting disease associations due to linkage disequilibrium using haplotype tags: a class of tests and the determinants of statistical power. *Hum.Hered.* 56 (1-3):18-31.

54. Chen CJ et al (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 47 (3):381-389.

55. Chen G et al (2003) Human gastrointestinal sulfotransferases: identification and distribution. *Toxicol.Appl.Pharmacol.* 187 (3):186-197.

56. Chew CN, Nolan DJ, and Jewell DP (1991) Small bowel gas in severe ulcerative colitis. *Gut* 32 (12):1535-1537.

57. Chianale J et al (1995) Differences between nuclear run-off and mRNA levels for multidrug resistance gene expression in the cephalocaudal axis of the mouse intestine. *Biochim Biophys Acta* 1264 (3):369-376.

58. Chikanza IC and Kozaci DL (2004) Corticosteroid resistance in rheumatoid arthritis: molecular and cellular perspectives. *Rheumatology.(Oxford)* 43 (11):1337-1345.

59. Cho JH et al (1998b) Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc.Natl.Acad.Sci.U.S.A* 95 (13):7502-7507.

60. Cho JH et al (1998a) Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc.Natl.Acad.Sci.U.S.A* 95 (13):7502-7507.

61. Chrencik JE et al (2005) Structural disorder in the complex of human pregnane x receptor and the macrolide antibiotic rifampicin. *Mol.Endocrinol.* 19 (5):1125-1134.

62. Cima RR and Pemberton JH (2004) Protagonist: Early surgical intervention in ulcerative colitis. *Gut* 53 (2):306-307.

63. Cohen RD, Brodsky AL, and Hanauer S (1999) A comparison of quality of life in patients with severe ulcerative colitis preoperatively and postoperatively. *Inflamm Bowel Dis* 5:1-10.

64. Cohen RD, Stein R, and Hanauer SB (1999) Intravenous cyclosporin in ulcerative colitis: a five-year experience. *Am J Gastroenterol* 94 (6):1587-1592.

65. Collie-Duguid ES et al (1999) The frequency and distribution of thiopurine methyltransferase alleles in Caucasian and Asian populations. *Pharmacogenetics JID - 9211735* 9 (1):37-42.

66. Colombel JF et al (2000) Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* 118 (6):1025-1030.

67. Cordon-Cardo C et al (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci U S A* 86 (2):695-698.

68. Creed TJ et al (2003) Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroid-resistant ulcerative colitis. *Aliment Pharmacol Ther* 18 (1):65-75.

69. Croucher PJ et al (2003) Lack of association between the C3435T MDR1 gene polymorphism and inflammatory bowel disease in two independent Northern European populations. *Gastroenterology* 125 (6):1919-1920.

70. Cummings JR et al (2005) Oral methotrexate in ulcerative colitis. *Aliment.Pharmacol.Ther.* 21 (4):385-389.

71. Cuthbert AP et al (2002) The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology* 122 (4):867-874.

72. D'Haens G et al (2001) Intravenous cyclosporine versus intravenous corticosteroids as single therapy for severe attacks of ulcerative colitis. *Gastroenterology* 120 (6):1323-1329.

73. Daly MJ et al (2001) High-resolution haplotype structure in the human genome. *Nat Genet* 29 (2):229-232.

74. de Bakker PI et al (2005) Efficiency and power in genetic association studies. *Nat.Genet.* 37 (11):1217-1223.

75. de la Concha EG et al (2000) Susceptibility to severe ulcerative colitis is associated with polymorphism in the central MHC gene IKBL. *Gastroenterology* 119 (6):1491-1495.

76. Deleuze JF et al (1996) Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. *Hepatology* 23 (4):904-908.

77. Demeule M et al (1999) Dexamethasone modulation of multidrug transporters in normal tissues. *FEBS Lett* 442 (2-3):208-214.

78. DeRijk RH et al (2002) Glucocorticoid receptor variants: clinical implications. *Journal of Steroid Biochemistry and Molecular Biology* 81:103-122.

79. Dietrich CG, Geier A, and Oude Elferink RP (2003) ABC of oral bioavailability: transporters as gatekeepers in the gut. *Gut* 52 (12):1788-1795.

80. Ding JW et al (1993) The role of bile and bile acids in bacterial translocation in obstructive jaundice in rats. *Eur.Surg.Res.* 25 (1):11-19.

81. Ding X and Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu.Rev.Pharmacol.Toxicol.* 43:149-173.

82. Dotan I et al (2001) Low dose low-molecular weight heparin (enoxaparin) is effective as an adjuvant treatment in active ulcerative colitis: an open trial. *Dig Dis* 46:2239-2244.

83. Drescher S et al (2002) MDR1 gene polymorphisms and disposition of the P-glycoprotein substrate fexofenadine. *Br J Clin Pharmacol* 53 (5):526-534.

84. Dring MM et al (2006) The Pregnane X Receptor Locus Is Associated With Susceptibility to Inflammatory Bowel Disease. *Gastroenterology* 130 (2):341-348.

85. Duchmann R et al (1999) T cell specificity and cross reactivity towards enterobacteria, bacteroides, bifidobacterium, and antigens from resident intestinal flora in humans. *Gut* 44 (6):812-818.

86. Duerr RH et al (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 5804:1461-1463.

87. Duerr RH et al (1998) Linkage and association between inflammatory bowel disease and a locus on chromosome 12. *Am J Hum Genet JID - 0370475* 63 (1):95-100.

88. Duerr RH et al (2000b) High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. *Am.J.Hum.Genet.* 66 (6):1857-1862.

89. Duerr RH et al (2000a) High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. *Am.J.Hum.Genet.* 66 (6):1857-1862.

90. Duerr, R. H. and Chesny IJ. Associations between HLA-DR alleles and subsets of ulcerative colitis defined by extent of colitis. Gastroenterology (113). 2-7-1997.

91. Eaden J et al (2000) Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment.Pharmacol.Ther.* 14 (2):145-153.

92. Eaden JA and Mayberry JF (2002) Guidelines for screening and surveillance of asymptomatic colorectal cancer in patients with inflammatory bowel disease. *Gut* 51 Suppl 5:V10-V12.

93. Edwards F.C. and Truelove SC (1963) The course and prognosis of ulcerative colitis. *Gut* 4:299-315.

94. Ekbom A et al (1990) Ulcerative colitis and colorectal cancer. A population-based study. *N.Engl.J Med.* 323 (18):1228-1233.

95. Esworthy RS et al (2001) Mice with combined disruption of Gpx1 and Gpx2 genes have colitis. *Am.J.Physiol Gastrointest.Liver Physiol* 281 (3):G848-G855.

96. Esworthy RS et al (2003) Microflora trigger colitis in mice deficient in selenium-dependent glutathione peroxidase and induce Gpx2 gene expression. *Biol.Chem.* 384 (4):597-607.

97. Evans WE et al (1991) Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. *J Pediatr* 119 (6):985-989.

98. Fardel O et al (2001) Regulation of biliary drug efflux pump expression by hormones and xenobiotics. *Toxicology* 167 (1):37-46.

99. Farmer RG, Easley KA, and Rankin GB (1993) Clinical patterns, natural history, and progression of ulcerative colitis. A long-term follow-up of 1116 patients. *Dig Dis Sci* 38 (6):1137-1146.

100. Farrell RJ and Kelleher D (2003) Glucocorticoid resistance in inflammatory bowel disease. *J.Endocrinol.* 178 (3):339-346.

101. Farrell RJ et al (2002) P-Glycoprotein-170 inhibition significantly reduces cortisol and ciclosporin efflux from human intestinal epithelial cells and T lymphocytes. *Aliment Pharmacol Ther* 16 (5):1021-1031.

102. Farrell RJ et al (2000) High multidrug resistance (P-glycoprotein 170) expression in inflammatory bowel disease patients who fail medical therapy. *Gastroenterology* 118 (2):279-288.

103. Faubion WAJ et al (2001) The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 121 (2):255-260.

104. Feagan BG et al (2000) A comparison of methotrexate with placebo for the maintenance of remission in Crohn's disease. North American Crohn's Study Group Investigators. *N Engl J Med* 342 (22):1627-1632.

105. Feagan BG et al (2005) Treatment of ulcerative colitis with a humanized antibody to the alpha4beta7 integrin. *N.Engl.J Med.* 352 (24):2499-2507.

106. Feagan BG et al (1995) Methotrexate for the treatment of Crohn's disease. The North American Crohn's Study Group Investigators. *N Engl J Med JID - 0255562* 332 (5):292-297.

107. Fellay J et al (2002) Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet JID - 2985213R* 359 (9300):30-36.

108. Fiocchi, C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 115, 182-205. 1998.

109. Fockens P et al (1995) Comparison of the efficacy and safety of 1.5 compared with 3.0 g oral slow-release mesalazine (Pentasa) in the maintenance treatment of ulcerative colitis. Dutch Pentasa Study Group. *Eur J Gastroenterol Hepatol* 7 (11):1025-1030.

110. Fojo AT et al (1987) Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci U S A* 84 (1):265-269.

111. Fonti R et al (1998) Carbonic anhydrase I reduction in colonic mucosa of patients with active ulcerative colitis. *Dig.Dis.Sci.* 43 (9):2086-2092.

112. Fraser AG, Orchard TR, and Jewell DP (2002) The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. *Gut* 50 (4):485-489.

113. Fricker G et al (1996) Relevance of p-glycoprotein for the enteral absorption of cyclosporin A: in vitro-in vivo correlation. *Br J Pharmacol* 118 (7):1841-1847.

114. Furuno T et al (2002) Expression polymorphism of the blood-brain barrier component P-glycoprotein (MDR1) in relation to Parkinson's disease. *Pharmacogenetics* 12 (7):529-534.

115. Fuss IJ et al (1996) Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol.* 157 (3):1261-1270.

116. Futami S et al (1995) HLA-DRB1*1502 allele, subtype of DR15, is associated with susceptibility to ulcerative colitis and its progression. *Dig Dis Sci* 40 (4):814-818.

117. Gabriel SB et al (2002) The structure of haplotype blocks in the human genome. *Science* 296 (5576):2225-2229.

118. Gadsby DC, Vergani P, and Csanady L (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature* 440 (7083):477-483.

119. Gaikovitch EA et al (2003) Polymorphisms of drug-metabolizing enzymes CYP2C9, CYP2C19, CYP2D6, CYP1A1, NAT2 and of P-glycoprotein in a Russian population. *Eur.J.Clin.Pharmacol.* 59 (4):303-312.

120. Gasche C et al (2000) A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm Bowel Dis* 6 (1):8-15.

121. Gaya, D. R., Russell R.K., Nimmo E.R., and Satsangi, J. New genes in inflammatory bowel disease: lessons for complex diseases? Lancet 367, 1271-1284. 2006.

122. Geick A, Eichelbaum M, and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 276 (18):14581-14587.

123. George J et al (1996) The long-term outcome of ulcerative colitis treated with 6-mercaptopurine. *Am J Gastroenterol* 91 (9):1711-1714.

124. Gerloff T et al (2002) MDR1 genotypes do not influence the absorption of a single oral dose of 1 mg digoxin in healthy white males. *Br.J.Clin.Pharmacol.* 54 (6):610-616.

125. Gewirtz AT et al (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 167 (4):1882-1885.

126. Giallourakis C et al (2003) IBD5 is a General Risk Factor for Inflammatory Bowel Disease: Replication of Association with Crohn Disease and Identification of a Novel Association with Ulcerative Colitis. *Am J Hum Genet* 73 (1):205-211.

127. Gionchetti P et al (2006) Antibiotics and probiotics in treatment of inflammatory bowel disease. *World J Gastroenterol* 12 (21):3306-3313.

128. Glas J et al (2004) MDR1 gene polymorphism in ulcerative colitis. *Gastroenterology* 126 (1):367.

129. Goldstein DB et al (2003) Genome scans and candidate gene approaches in the study of common diseases and variable drug responses. *Trends Genet.* 19 (11):615-622.

130. Gonzalez-Huix F et al (1993) Polymeric enteral diets as primary treatment of active Crohn's disease: a prospective steroid controlled trial. *Gut* 34 (6):778-782.

131. Greiner B et al (1999) The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. *J Clin Invest* 104 (2):147-153.

132. Groux H et al (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389 (6652):737-742.

133. Gu X et al (2006a) Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J.Biol.Chem.* 281 (26):17882-17889.

134. Gu X et al (2006b) Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J.Biol.Chem.* 281 (26):17882-17889.

135. H.Blokzijl, S. Vander Borght L. Libbrecht L. I. Bok F. A. van den Heuvel G. Dijkstra H. Moshage T. A. Roskams P. L. Jansen K. Faber. Decreased Multidrug Resistance Protein 1 (mdr1) Expression in Inflamed Human Intestinal Epithelium. Gastroenterology , 1624. 1-5-2005.

136. Halfvarson J et al (2003) Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. *Gastroenterology* 124 (7):1767-1773.

137. Halstensen TS, Das KM, and Brandtzaeg P (1995) Epithelial deposits of immunoglobulin G1 and activated complement co-localize with the "M(r) 40kD" putative auto-antigen in ulcerative colitis. *Adv.Exp.Med.Biol.* 371B:1273-1276.

138. Hamid Q et al (1999) Increased glucocorticoid receptor b in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 159:1600-1604.

139. Hamilos DL et al (2001) GRbeta expression in nasal polyp inflammatory cells and its relationship to the anti-inflammatory effects of intranasal flucatisone. *J Allergy Clin Immunol* 105:782-787.

140. Hampe J et al (2001) Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet JID - 2985213R* 357 (9272):1925-1928.

141. Hampe J et al (1999a) A genomewide analysis provides evidence for novel linkages in inflammatory bowel disease in a large European cohort. *Am.J.Hum.Genet.* 64 (3):808-816.

142. Hampe J et al (1999b) Linkage of inflammatory bowel disease to human chromosome 6p. *Am J Hum Genet* 65 (6):1647-1655.

143. Hanauer SB (2005) Top-down versus step-up approaches to chronic inflammatory bowel disease: presumed innocent or presumed guilty. *Nat.Clin.Pract.Gastroenterol.Hepatol.* 2 (11):493.

144. Hanauer SB (2006) Review article: high-dose aminosalicylates to induce and maintain remissions in ulcerative colitis. *Aliment.Pharmacol.Ther.* 24 Suppl 3:37-40.

145. Hanauer SB et al (2002) Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet JID - 2985213R* 359 (9317):1541-1549.

146. Hanauer SB et al (2005) Delayed-release oral mesalamine at 4.8 g/day (800 mg tablet) for the treatment of moderately active ulcerative colitis: the ASCEND II trial. *Am.J Gastroenterol* 100 (11):2478-2485.

147. Hawthorne AB et al (1992) Randomised controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ* 305 (6844):20-22.

148. Hearing SD et al (1999) Predicting therapeutic outcome in severe ulcerative colitis by measuring in vitro steroid sensitivity of proliferating peripheral blood lymphocytes. *Gut* 45 (3):382-388.

149. Helio T et al (2003) CARD15/NOD2 gene variants are associated with familially occurring and complicated forms of Crohn's disease. *Gut* 52 (4):558-562.

150. Heuschen UA et al (2001a) One- or two-stage procedure for restorative proctocolectomy: rationale for a surgical strategy in ulcerative colitis. *Ann.Surg.* 234 (6):788-794.

151. Heuschen UA et al (2001b) Backwash ileitis is strongly associated with colorectal carcinoma in ulcerative colitis. *Gastroenterology* 120 (4):841-847.

152. Heuschkel RB (2000) Enteral nutrition in children with Crohn's disease. *J.Pediatr.Gastroenterol.Nutr.* 31 (5):575.

153. Hill W.G. and Robertson A. Linkage disequilibrium between SNPs was measured using r^2. Theo.Appl.Genet. 38, 226-331. 1968.

154. Hitzl M et al (2001) The C3435T mutation in the human MDR1 gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. *Pharmacogenetics JID - 9211735* 11 (4):293-298.

155. Ho GT et al (2006a) The efficacy of corticosteroid therapy in inflammatory bowel disease: analysis of a 5-year UK inception cohort. *Aliment.Pharmacol.Ther.* 24 (2):319-330.

156. Ho GT, Gaya DR, and Satsangi J (2005) Multidrug resistance (MDR1) gene in inflammatory bowel disease: a key player? *Inflamm.Bowel Dis* 11 (11):1013-1019.

157. Ho GT, Moodie FM, and Satsangi J (2003) Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? *Gut* 52 (5):759-766.

158. Ho GT et al (2004) Predicting the outcome of severe ulcerative colitis: development of a novel risk score to aid early selection of patients for second-line medical therapy or surgery. *Aliment.Pharmacol.Ther.* 19 (10):1079-1087.

159. Ho GT et al (2005) Allelic variations of the multidrug resistance gene determine susceptibility and disease behavior in ulcerative colitis. *Gastroenterology* 128 (2):288-296.

160. Ho GT et al (2006b) ABCB1/MDR1 gene determines susceptibility and phenotype in ulcerative colitis: discrimination of critical variants using a gene-wide haplotype tagging approach. *Hum.Mol.Genet.* 15 (5):797-805.

161. Ho GT et al (2006c) Lack of association of the pregnane X receptor (PXR/NR1I2) gene with inflammatory bowel disease: parallel allelic association study and gene wide haplotype analysis. *Gut* 55 (11):1676-1677.

162. Hoffmeyer S et al (2000a) Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc Natl Acad Sci U S A* 97 (7):3473-3478.

163. Hoffmeyer S et al (2000b) Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-

glycoprotein expression and activity in vivo. *Proc Natl Acad Sci U S A* 97 (7):3473-3478.

164. Hogenauer C et al (2003) Effect of oral tacrolimus (FK 506) on steroid-refractory moderate/severe ulcerative colitis. *Aliment.Pharmacol.Ther.* 18 (4):415-423.

165. Hommes DW, D'Haens, G., Baert, F., and et al. Management of recent onset Crohn's disease-a controlled randomized trial comparing step-up and top-down therapy. Gastroenterology . 2005.

166. Honda M et al (2000) Expression of glucocorticoid receptor beta in lymphocytes of patients woth glucocorticoid-resistant ulcerative colitis. *Gastroenterology* 118:859-866.

167. Horinouchi M et al (2002) Significant genetic linkage of MDR1 polymorphisms at positions 3435 and 2677: functional relevance to pharmacokinetics of digoxin. *Pharm.Res.* 19 (10):1581-1585.

168. Hsu SI, Lothstein L, and Horwitz SB (1989) Differential overexpression of three mdr gene family members in multidrug-resistant J774.2 mouse cells. Evidence that distinct P-glycoprotein precursors are encoded by unique mdr genes. *J Biol Chem* 264 (20):12053-12062.

169. Hugot JP et al (2001a) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411 (6837):599-603.

170. Hugot JP et al (2001b) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411 (6837):599-603.

171. Hugot JP et al (2001c) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411 (6837):599-603.

172. Hugot JP et al (1996b) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379 (6568):821-823.

173. Hugot JP et al (1996a) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379 (6568):821-823.

174. Hyams, J. S., Markowitz, J. S., Griffiths, A., and et al. The natural therapy of corticosteroid therapy for ulcerative colitis in children. Gastroenterology , M1157. 2005.

175. Hyde GM and Jewell DP (1997) Review article: the management of severe ulcerative colitis. *Aliment Pharmacol Ther* 11 (3):419-424.

176. Iizasa H et al (2003) Altered expression and function of P-glycoprotein in dextran sodium sulfate-induced colitis in mice. *J.Pharm.Sci.* 92 (3):569-576.

177. Inagaki T et al (2006) Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc.Natl.Acad.Sci.U.S.A* 103 (10):3920-3925.

178. Inoue N et al (2002) Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 123 (1):86-91.

179. Inoue S et al (1999) Characterization of cytokine expression in the rectal mucosa of ulcerative colitis: correlation with disease activity. *Am.J Gastroenterol* 94 (9):2441-2446.

180. Ito K et al (2006) Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J.Exp.Med.* 203 (1):7-13.

181. Jacquemin E (2000) Progressive familial intrahepatic cholestasis. Genetic basis and treatment. *Clin Liver Dis* 4 (4):753-763.

182. Jarnerot G et al (2005b) Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 128 (7):1805-1811.

183. Jarnerot G et al (2005a) Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 128 (7):1805-1811.

184. Jarnerot G, Rolny P, and Sandberg-Gertzen H (1985) Intensive intravenous treatment of ulcerative colitis. *Gastroenterology* 89 (5):1005-1013.

185. Jayaram H, Satsangi J, and Chapman RW (2001) Increased colorectal neoplasia in chronic ulcerative colitis complicated by primary sclerosing cholangitis: fact or fiction? *Gut JID* 48 (3):430-434.

186. Jewell DP and Truelove SC (1974) Azathioprine in ulcerative colitis: final report on controlled therapeutic trial. *Br.Med.J.* 4 (5945):627-630.

187. Johne A et al (2002a) Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene. *Clin.Pharmacol.Ther.* 72 (5):584-594.

188. Johne A et al (2002b) Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene. *Clin.Pharmacol.Ther.* 72 (5):584-594.

189. Johnson GC et al (2001) Haplotype tagging for the identification of common disease genes. *Nat.Genet.* 29 (2):233-237.

190. Johnson P et al (2004) Female infertility after ileal pouch-anal anastomosis for ulcerative colitis. *Dis.Colon Rectum* 47 (7):1119-1126.

191. Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 455 (1):152-162.

192. Kamatani N et al (2004) Large-scale single-nucleotide polymorphism (SNP) and haplotype analyses, using dense SNP Maps, of 199 drug-related genes in 752 subjects: the analysis of the association between uncommon SNPs within haplotype blocks and the haplotypes constructed with haplotype-tagging SNPs. *Am.J.Hum.Genet.* 75 (2):190-203.

193. Kamm MA (2004) Antagonist: Early surgical intervention in ulcerative colitis. *Gut* 53 (2):308-309.

194. Karlsen T.H. et al (2006) Polymorphisms in the Steroid and Xenobiotic Receptor Gene Influence Survival in Primary Sclerosing Cholangitis. *Gastroenterology* 131:781-787.

195. Katakura K et al (2005) Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J Clin.Invest* 115 (3):695-702.

196. Ke X et al (2005) A comparison of tagging methods and their tagging space. *Hum.Mol.Genet.* 14 (18):2757-2767.

197. Kim RB et al (2001b) Identification of functionally variant MDR1 alleles among European Americans and African Americans. *Clin Pharmacol Ther* 70 (2):189-199.

198. Kim RB et al (2001a) Identification of functionally variant MDR1 alleles among European Americans and African Americans. *Clin Pharmacol Ther* 70 (2):189-199.

199. Kimchi-Sarfaty C, Gribar JJ, and Gottesman MM (2002) Functional characterization of coding polymorphisms in the human MDR1 gene using a vaccinia virus expression system. *Mol Pharmacol* 62 (1):1-6.

200. Kirk AP and Lennard-Jones JE (1982) Controlled trial of azathioprine in chronic ulcerative colitis. *Br.Med.J (Clin.Res.Ed)* 284 (6325):1291-1292.

201. Klein W et al (2001) The polymorphism at position -174 of the IL-6 gene is not associated with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 13 (1):45-47.

202. Kliewer SA, Goodwin B, and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr.Rev.* 23 (5):687-702.

203. Klimecki WT et al (1994) P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* 83 (9):2451-2458.

204. Koss K et al (2000) Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies. *Genes Immun* 1 (3):185-190.

205. Koutroubakis IE and Vlachonikolis IG (2000) Appendectomy and the development of ulcerative colitis: results of a metaanalysis of published case-control studies. *Am.J Gastroenterol* 95 (1):171-176.

206. Kullak-Ublick GA, Stieger B, and Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* 126 (1):322-342.

207. Labialle S et al (2002) Transcriptional regulators of the human multidrug resistance 1 gene: recent views. *Biochem Pharmacol* 64 (5-6):943-948.

208. Laine L and Hanauer SB (2003) Considerations in the management of steroid-dependent Crohn's disease. *Gastroenterology* 125 (3):906-910.

209. Lala S et al (2003) Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* 125 (1):47-57.

210. Lamers CB et al (1999) Azathioprine: an update on clinical efficacy and safety in inflammatory bowel disease. *Scan J Gastroenterology Suppl* (230):111-115.

211. Lane SJ et al (1996) Hypothalamic-pituitary-adrenal axis in corticosteroid-resistant bronchial asthma. *Am.J.Respir.Crit Care Med.* 153 (2):557-560.

212. Langholz E et al (1994) Course of ulcerative colitis: analysis of changes in disease activity over years. *Gastroenterology JID - 0374630* 107 (1):3-11.

213. Langmann T et al (2003) Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. *Clin.Chem.* 49 (2):230-238.

214. Langmann T et al (2004) Loss of detoxification in inflammatory bowel disease: Dysregulation of pregnane X receptor target genes. *Gastroenterology* 127 (1):26-40.

215. Langmead L et al (2004) Randomized, double-blind, placebo-controlled trial of oral aloe vera gel for active ulcerative colitis. *Aliment.Pharmacol.Ther.* 19 (7):739-747.

216. Lashner BA et al (1997) The effect of folic acid supplementation on the risk for cancer or dysplasia in ulcerative colitis. *Gastroenterology* 112 (1):29-32.

217. Lennard L (2002) TPMT in the treatment of Crohn's disease with azathioprine. *Gut* 51 (2):143-146.

218. Lennard-Jones JE (1989) Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 170:2-6.

219. Lennard-Jones JE et al (1975) Assessment of severity in colitis: a preliminary study. *Gut* 16 (8):579-584.

220. Lennard-Jones JE and Shivananda S (1997) Clinical uniformity of inflammatory bowel disease a presentation and during the first year of disease in the north and south of Europe. EC-IBD Study Group. *Eur J Gastroenterol Hepatol* 9 (4):353-359.

221. Leong RW et al (2003) NOD2/CARD15 gene polymorphisms and Crohn's disease in the Chinese population. *Aliment.Pharmacol.Ther.* 17 (12):1465-1470.

222. Lepisto A, Luukkonen P, and Jarvinen HJ (2002) Cumulative failure rate of ileal pouch-anal anastomosis and quality of life after failure. *Dis.Colon Rectum* 45 (10):1289-1294.

223. Lesage S et al (2002) CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am.J.Hum.Genet.* 70 (4):845-857.

224. Leung DY et al (2000) Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* (162):1-3.

225. Libioulle C et al (2007) Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS.Genet.* 3 (4):e58.

226. Lichtiger S et al (1994) Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 330 (26):1841-1845.

227. Lin JH et al (1999) Effect of dexamethasone on the intestinal first-pass metabolism of indinavir in rats: evidence of cytochrome P-450 3A [correction of P-450 A] and p-glycoprotein induction. *Drug Metab Dispos* 27 (10):1187-1193.

228. Lindberg E et al (1988) Smoking and inflammatory bowel disease. A case control study. *Gut* 29 (3):352-357.

229. Lindgren SC et al (1998) Early predictors of glucocorticosteroid treatment failure in severe and moderately severe attacks of ulcerative colitis. *Eur J Gastroenterol Hepatol* 10 (10):831-835.

230. Liu Z et al (2000) Prevention of experimental colitis in SCID mice reconstituted with CD45RBhigh CD4+ T cells by blocking the CD40-CD154 interactions. *J Immunol.* 164 (11):6005-6014.

231. Lodes MJ et al (2004) Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin.Invest* 113 (9):1296-1306.

232. Loftus EV, Jr. (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 126 (6):1504-1517.

233. Loguercio C et al (2003) Gluthathione supplementation improves oxidative damage in experimental colitis. *Dig Liver Dis* 35:635-641.

234. Lohmuller JL et al (1990) Pouchitis and extraintestinal manifestations of inflammatory bowel disease after ileal pouch-anal anastomosis. *Ann.Surg.* 211 (5):622-627.

235. Lorenzo-Zuniga V et al (2006) Insulin-like growth factor I improves intestinal barrier function in cirrhotic rats. *Gut* 55 (9):1306-1312.

236. Louis E et al (1996) Cytokine gene polymorphisms in inflammatory bowel disease. *Gut* 39 (5):705-710.

237. Lu K et al (2001) Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. *Am.J.Hum.Genet.* 69 (2):278-290.

238. Ludescher C et al (1998) Differential activity of P-glycoprotein in normal blood lymphocyte subsets. *Br J Haematol* 101 (4):722-727.

239. Ma Y et al (1999a) A genome-wide search identifies potential new susceptibility loci for Crohn's disease. *Inflamm Bowel Dis* 5 (4):271-278.

240. Ma Y et al (1999b) A genome-wide search identifies potential new susceptibility loci for Crohn's disease. *Inflamm.Bowel.Dis.* 5 (4):271-278.

241. Mackey AC et al (2007) Hepatosplenic T cell lymphoma associated with infliximab use in young patients treated for inflammatory bowel disease. *J Pediatr.Gastroenterol Nutr.* 44 (2):265-267.

242. Maggio-Price L et al (2005) Dual Infection with Helicobacter bilis and Helicobacter hepaticus in P-Glycoprotein-Deficient mdr1a-/- Mice Results in Colitis that Progresses to Dysplasia. *Am.J.Pathol.* 166 (6):1793-1806.

243. Maggio-Price L et al (2002) Helicobacter bilis infection accelerates and H. hepaticus infection delays the development of colitis in multiple drug resistance-deficient (mdr1a-/-) mice. *Am J Pathol* 160 (2):739-751.

244. Maillefert JF et al (1996) Expression of the multidrug resistance glycoprotein 170 in the peripheral blood lymphocytes of rheumatoid arthritis patients. The

percentage of lymphocytes expressing glycoprotein 170 is increased in patients treated with prednisolone. *Br J Rheumatol* 35 (5):430-435.

245. Mansfield JC et al (1994) Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. *Gastroenterology* 106 (3):637-642.

246. Marteau P et al (2005) Combined oral and enema treatment with Pentasa (mesalazine) is superior to oral therapy alone in patients with extensive mild/moderate active ulcerative colitis: a randomised, double blind, placebo controlled study. *Gut* 54 (7):960-965.

247. Martensson J, Jain A, and Meister A (1990) Glutathione is required for intestinal function. *Proc.Natl.Acad.Sci.U.S.A* 87 (5):1715-1719.

248. Matthews JG et al (2004) Defective glucocorticoid receptor nuclear translocation and altered histone acetylation patterns in glucocorticoid-resistant patients. *J.Allergy Clin.Immunol.* 113 (6):1100-1108.

249. Mayer U et al (1996) Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *Br J Pharmacol* 119 (5):1038-1044.

250. McGovern, D., Ahmad, T., Van Heel, D., and Jewell, D. A genetic panel strongly predicts the need for colectomy un ulcerative colitis. Gastroenterology A525. 1-4-2004.

251. McGovern DP et al (2005) Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. *Hum.Mol.Genet.* 14 (10):1245-1250.

252. Merrett MN et al (1996) Smoking may prevent pouchitis in patients with restorative proctocolectomy for ulcerative colitis. *Gut* 38 (3):362-364.

253. Mickley LA et al (1997) Gene rearrangement: a novel mechanism for MDR-1 gene activation. *J Clin Invest* 99 (8):1947-1957.

254. Mirza MM et al (2003) Genetic evidence for interaction of the 5q31 cytokine locus and the CARD15 gene in Crohn disease. *Am.J.Hum.Genet.* 72 (4):1018-1022.

255. Mizoguchi A, Mizoguchi E, and Bhan AK (1999) The critical role of interleukin 4 but not interferon gamma in the pathogenesis of colitis in T-cell receptor alpha mutant mice. *Gastroenterology* 116 (2):320-326.

256. Mizoguchi E et al (2003) Colonic epithelial functional phenotype varies with type and phase of experimental colitis. *Gastroenterology* 125 (1):148-161.

257. Monsen U et al (1991) Prevalence of inflammatory bowel disease among relatives of patients with Crohn's disease. *Scand.J.Gastroenterol.* 26 (3):302-306.

258. Monsuur AJ et al (2005) Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat.Genet.* 37 (12):1341-1344.

259. Montano E et al (1996) P-glycoprotein expression in circulating blood leukocytes of patients with steroid-resistant asthma. *J Investig Allergol Clin Immunol* 6 (1):14-21.

260. Moodie, F. M., Lyons V, Satsangi, J., and Seckl J. Effects of glucocorticoids on expression of P-glycoprotein and Glucocorticoid receptor in the intestinal epithelium. Gastroenterology M1136. 2004.

261. Moodie, F. M., Noble J, Satsangi, J., and Seckl J. Glucocorticoid access and action in the rat colon: expression and regulation of multidrug resistance 1a gene (mdr1a), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and 11-beta-hydroxysteroid dehydrogenase type 2 (11BHDS2). Gut JID - 2985108R 197(A54). 1-4-2003.

262. Morita N, Yasumori T, and Nakayama K (2003) Human MDR1 polymorphism: G2677T/A and C3435T have no effect on MDR1 transport activities. *Biochem.Pharmacol.* 65 (11):1843-1852.

263. Moum B et al (1997) Clinical course during the 1st year after diagnosis in ulcerative colitis and Crohn's disease. Results of a large, prospective population-based study in southeastern Norway, 1990-93. *Scand.J.Gastroenterol.* 32 (10):1005-1012.

264. Munkholm P et al (1994) Frequency of glucocorticoid resistance and dependency in Crohn's disease. *Gut* 5 (3):360-362.

265. Murakami T et al (2002) Factors affecting the expression and function of P-glycoprotein in rats: drug treatments and diseased states. *Pharmazie* 57 (2):102-107.

266. Mutch DM et al (2004) Regional variations in ABC transporter expression along the mouse intestinal tract. *Physiol Genomics* 17 (1):11-20.

267. Nagata K et al (2000) Nonequivalent nucleotide trapping in the two nucleotide binding folds of the human multidrug resistance protein MRP1. *J Biol Chem* 275 (23):17626-17630.

268. Nakamura T et al (2002) Effect of the mutation (C3435T) at exon 26 of the MDR1 gene on expression level of MDR1 messenger ribonucleic acid in duodenal enterocytes of healthy Japanese subjects. *Clin Pharmacol Ther* 71 (4):297-303.

269. Navazo L et al (2001) Oral microemulsion cyclosporine in the treatment of steroid-refractory attacks of ulcerative and indeterminate colitis. *Scand.J Gastroenterol* 36 (6):610-614.

270. Neale BM and Sham PC (2004) The future of association studies: gene-based analysis and replication. *Am.J.Hum.Genet.* 75 (3):353-362.

271. Negoro K et al (2003) Analysis of the IBD5 locus and potential gene-gene interactions in Crohn's disease. *Gut* 52 (4):541-546.

272. Neish AS et al (2000) Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289 (5484):1560-1563.

273. Neurath MF et al (1996) Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. *J Exp.Med.* 183 (6):2605-2616.

274. Newman B et al (2005) A risk haplotype in the Solute Carrier Family 22A4/22A5 gene cluster influences phenotypic expression of Crohn's disease. *Gastroenterology* 128 (2):260-269.

275. Nicholls RJ (2002) Review article: ulcerative colitis--surgical indications and treatment. *Aliment.Pharmacol.Ther.* 16 Suppl 4:25-28.

276. Noble CL et al (2005) The contribution of OCTN1/2 variants within the IBD5 locus to disease susceptibility and severity in Crohn's disease. *Gastroenterology* 129 (6):1854-1864.

277. Ogura Y et al (2001a) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411 (6837):603-606.

278. Ogura Y et al (2001b) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411 (6837):603-606.

279. Olavesen MG et al (2000) Analysis of single-nucleotide polymorphisms in the interleukin-4 receptor gene for association with inflammatory bowel disease. *Immunogenetics* 51 (1):1-7.

280. Oren R et al (1996) Methotrexate in chronic active ulcerative colitis: a double-blind, randomized, Israeli multicenter trial. *Gastroenterology* 110 (5):1416-1421.

281. Oresland T et al (1990) A prospective randomized comparison of two different pelvic pouch designs. *Scand.J Gastroenterol* 25 (10):986-996.

282. Orholm M et al (2000) Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand.J.Gastroenterol.* 35 (10):1075-1081.

283. Orholm M et al (1991) Familial occurrence of inflammatory bowel disease. *N Engl J Med* 324 (2):84-88.

284. Orth T et al (2000) Mycophenolate mofetil versus azathioprine in patients with chronic active ulcerative colitis: a 12-month pilot study. *Am.J Gastroenterol* 95 (5):1201-1207.

285. Pace DE et al (2002) Early experience with laparoscopic ileal pouch-anal anastomosis for ulcerative colitis. *Surg.Laparosc.Endosc.Percutan.Tech.* 12 (5):337-341.

286. Pacifici GM et al (1988) Tissue distribution of drug-metabolizing enzymes in humans. *Xenobiotica* 18 (7):849-856.

287. Panes J et al (2000) Comparison of heparin and steroids in the treatment of moderate and severe ulcerative colitis. *Gastroenterology* 119 (4):903-908.

288. Panwala CM, Jones JC, and Viney JL (1998) A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *J Immunol JID - 2985117R* 161 (10):5733-5744.

289. Paoluzi OA et al (2005) Comparison of two different daily dosages (2.4 vs. 1.2 g) of oral mesalazine in maintenance of remission in ulcerative colitis patients: 1-year follow-up study. *Aliment.Pharmacol.Ther.* 21 (9):1111-1119.

290. Papo M et al (1996) Antineutrophil cytoplasmic antibodies in relatives of patients with inflammatory bowel disease. *Am.J Gastroenterol* 91 (8):1512-1515.

291. Parkes M et al (2007) Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat.Genet.* 39 (7):830-832.

292. Parkes M, Satsangi J, and Jewell D (1998b) Contribution of the IL-2 and IL-10 genes to inflammatory bowel disease (IBD) susceptibility. *Clin Exp Immunol* 113 (1):28-32.

293. Parkes M, Satsangi J, and Jewell D (1998a) Contribution of the IL-2 and IL-10 genes to inflammatory bowel disease (IBD) susceptibility. *Clin Exp Immunol* 113 (1):28-32.

294. Payne DN and Adcock IM (2001) Molecular mechanisms of corticosteroid actions. *Paediatr.Respir.Rev.* 2 (2):145-150.

295. Pearson DC et al (1995) Azathioprine and mercaptopurine in Crohn's disease: a meta-analysis. *Ann Intern Med* 123:132-142.

296. Pearson DC et al (2000) Azathioprine for maintaining remission of Crohn's disease. *Cochrane.Database.Syst.Rev.* (2):CD000067.

297. Peltekova VD et al (2004) Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat.Genet.* 36 (5):471-475.

298. Penna C et al (1996) Pouchitis after ileal pouch-anal anastomosis for ulcerative colitis occurs with increased frequency in patients with associated primary sclerosing cholangitis. *Gut* 38 (2):234-239.

299. Pinczowski D et al (1994) Risk factors for colorectal cancer in patients with ulcerative colitis: a case-control study. *Gastroenterology* 107 (1):117-120.

300. Podolsky DK (2002) Inflammatory bowel disease. *N.Engl.J.Med.* 347 (6):417-429.

301. Potocnik U et al (2004) Polymorphisms in multidrug resistance 1 (MDR1) gene are associated with refractory Crohn disease and ulcerative colitis. *Genes Immun.* 5 (7):530-539.

302. Present DH et al (1989) 6-Mercaptopurine in the management of inflammatory bowel disease: short- and long term toxicity. *Ann Intern Med* (111):641-649.

303. Probert CS et al (2003) Infliximab in moderately severe glucocorticoid resistant ulcerative colitis: a randomised controlled trial. *Gut* 52 (7):998-1002.

304. Probert CS et al (1993) Prevalence and family risk of ulcerative colitis and Crohn's disease: an epidemiological study among Europeans and south Asians in Leicestershire. *Gut* 34 (11):1547-1551.

305. Qin ZS, Niu T, and Liu JS (2002) Partition-ligation-expectation-maximization algorithm for haplotype inference with single-nucleotide polymorphisms. *Am.J.Hum.Genet.* 71 (5):1242-1247.

306. Quan VA et al (1997) Cyclosporin treatment for ulcerative colitis complicated by fatal Pneumocystis carinii pneumonia. *BMJ* 314 (7077):363-364.

307. Rao VV et al (1999) Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. *Proc Natl Acad Sci U S A* 96 (7):3900-3905.

308. Reinisch W et al (1995) Steroid dependency in Crohn's disease. *Lancet* 345 (8953):859-Reinisch, W.

309. Rhen T and Cidlowski JA (2005) Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N.Engl.J.Med.* 353 (16):1711-1723.

310. Riegler G et al (2000) Age-related clinical severity at diagnosis in 1705 patients with ulcerative colitis: a study by GISC (Italian Colon-Rectum Study Group). *Dig.Dis.Sci.* 45 (3):462-465.

311. Rioux JD et al (2001a) Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat.Genet.* 29 (2):223-228.

312. Rioux JD et al (2001b) Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat.Genet.* 29 (2):223-228.

313. Rioux JD et al (2000b) Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am.J.Hum.Genet.* 66 (6):1863-1870.

314. Rioux JD et al (2000a) Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am.J.Hum.Genet.* 66 (6):1863-1870.

315. Rioux JD et al (2000c) Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am.J.Hum.Genet.* 66 (6):1863-1870.

316. Rioux JD et al (2000d) Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am.J.Hum.Genet.* 66 (6):1863-1870.

317. Rioux JD et al (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat.Genet.*

318. Risch N and Merikangas K (1996) The future of genetic studies of complex human diseases. *Science* 273 (5281):1516-1517.

319. Ritchie JK, Powell-Tuck J, and Lennard-Jones JE (1978) Clinical outcome of the first ten years of ulcerative colitis and proctitis. *Lancet* 1 (8074):1140-1143.

320. Rockall TA et al (1996) Risk assessment after acute upper gastrointestinal haemorrhage. *Gut* 38 (3):316-321.

321. Roediger WE and Babidge W (1997) Human colonocyte detoxification. *Gut* 41 (6):731-734.

322. Rosenberg JL et al (1975) A controlled trial of azathioprine in the management of chronic ulcerative colitis. *Gastroenterology* 69 (1):96-99.

323. Rost D et al (2002) Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. *Am.J.Physiol Gastrointest.Liver Physiol* 282 (4):G720-G726.

324. Roth MP et al (1989) Geographic origins of Jewish patients with inflammatory bowel disease. *Gastroenterology* 97 (4):900-904.

325. Roussomoustakaki M et al (1997) Genetic markers may predict disease behavior in patients with ulcerative colitis. *Gastroenterology* 112 (6):1845-1853.

326. Rutgeerts P et al (2005) Infliximab for induction and maintenance therapy for ulcerative colitis. *N.Engl.J.Med.* 353 (23):2462-2476.

327. Rutter M et al (2004a) Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 126 (2):451-459.

328. Rutter MD et al (2004b) Cancer surveillance in longstanding ulcerative colitis: endoscopic appearances help predict cancer risk. *Gut* 53 (12):1813-1816.

329. Rutter MD et al (2006) Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis. *Gastroenterology* 130 (4):1030-1038.

330. Sagar PM et al (1992) Comparison of triplicated (S) and quadruplicated (W) pelvic ileal reservoirs. Studies on manovolumetry, fecal bacteriology, fecal volatile fatty acids, mucosal morphology, and functional results. *Gastroenterology* 102 (2):520-528.

331. Sakaeda T et al (2001) MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects. *Pharm.Res.* 18 (10):1400-1404.

332. Salphati L and Benet LZ (1998) Modulation of P-glycoprotein expression by cytochrome P450 3A inducers in male and female rat livers. *Biochem Pharmacol JID - 0101032* 55 (4):387-395.

333. Sandborn W et al (2000) Azathioprine or 6-mercaptopurine for inducing remission of Crohn's disease. *Cochrane.Database.Syst.Rev.* (2):CD000545.

334. Sandborn WJ (1998) Azathioprine: state of the art in inflammatory bowel disease. *Scand.J.Gastroenterol.Suppl* 225:92-99.

335. Sandborn WJ (2006) Preliminary data on the use of apheresis in inflammatory bowel disease. *Inflamm.Bowel.Dis.* 12 Suppl 1:S15-S21.

336. Sandborn WJ et al (1995) Antineutrophil cytoplasmic antibody correlates with chronic pouchitis after ileal pouch-anal anastomosis. *Am.J Gastroenterol* 90 (5):740-747.

337. Sandborn WJ et al (1996) Association of antineutrophil cytoplasmic antibodies with resistance to treatment of left-sided ulcerative colitis: results of a pilot study. *Mayo Clin.Proc.* 71 (5):431-436.

338. Sands BE et al (2004) Infliximab maintenance therapy for fistulizing Crohn's disease. *N.Engl.J.Med.* 350 (9):876-885.

339. Sartor RB (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat.Clin.Pract.Gastroenterol Hepatol* 3 (7):390-407.

340. Satsangi J et al (1996b) Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat Genet* 14 (2):199-202.

341. Satsangi J et al (1996c) Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat.Genet.* 14 (2):199-202.

342. Satsangi J et al (1996a) Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat.Genet.* 14 (2):199-202.

343. Saxon A et al (1990) A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. *J Allergy Clin.Immunol.* 86 (2):202-210.

344. Schinkel AH et al (1997) Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci U S A JID - 7505876* 94 (8):4028-4033.

345. Schreiber S et al (2005) Genetics of Crohn disease, an archetypal inflammatory barrier disease. *Nat.Rev.Genet.* 6 (5):376-388.

346. Schuetz EG et al (2001) Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J.Biol.Chem.* 276 (42):39411-39418.

347. Schwab M et al (2003a) An association between the C3435T MDR1 gene polymorphism and susceptibility for UC. *Gastroenterology* 124:26-33.

348. Schwab M et al (2003c) Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. *Gastroenterology* 124 (1):26-33.

349. Schwab M et al (2003b) Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. *Gastroenterology* 124 (1):26-33.

350. Schwab M et al (2002) Azathioprine therapy and adverse drug reactions in patients with inflammatory bowel disease: impact of thiopurine S-methyltransferase polymorphism. *Pharmacogenetics* 12 (6):429-436.

351. Schwer H et al (1997) Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem.Biophys.Res.Commun.* 233 (1):117-120.

352. Seki M et al (1998) Apoptosis of lymphocytes induced by glucocorticoids and relationship to therapeutic efficacy in patients with systemic lupus erythematosus. *Arthritis Rheum* 41:823-830.

353. Shapiro AB et al (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *Eur J Biochem* 259 (3):841-850.

354. Shomer NH et al (1998) Helicobacter bilis/Helicobacter rodentium co-infection associated with diarrhea in a colony of scid mice. *Lab Anim Sci* 48 (5):455-459.

355. Siddiqui A et al (2003) Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1. *N Engl J Med* 348 (15):1442-1448.

356. Sido B et al (1998) Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. *Gut* 42 (4):485-492.

357. Siegsmund M et al (2002) Association of the P-glycoprotein transporter MDR1(C3435T) polymorphism with the susceptibility to renal epithelial tumors. *J.Am.Soc.Nephrol.* 13 (7):1847-1854.

358. Silverberg MS et al (2005) Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can.J.Gastroenterol.* 19 Suppl A:5-36.

359. Sinclair TS, Brunt PW, and Mowat NA (1983) Nonspecific proctocolitis in northeastern Scotland: a community study. *Gastroenterology* 85 (1):1-11.

360. Sinha A et al (2003) Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis. *N.Engl.J Med.* 349 (4):350-357.

361. Slocum MM et al (1992) Absence of intestinal bile promotes bacterial translocation. *Am.Surg.* 58 (5):305-310.

362. Smit JJ et al (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 75 (3):451-462.

363. Smith BR et al (2004) Disease Location, Anti-Saccharomyces cerevisiae Antibody, and NOD2/CARD15 Genotype Influence the Progression of Disease Behavior in Crohn's Disease. *Inflamm Bowel Dis* 10 (5):521-528.

364. Soranzo N et al (2004) Identifying Candidate Causal Variants Responsible for Altered Activity of the ABCB1 Multidrug Resistance Gene. *Genome Res.* 14 (7):1333-1344.

365. Sousa AR et al (1999) In vivo resistance to corticosteroids in bronchial asthma is associated with enhanced phosyphorylation of JUN N-terminal kinase and failure of prednisolone to inhibit JUN N-terminal kinase phosphorylation. *J.Allergy Clin.Immunol.* 104 (3 Pt 1):565-574.

366. Sparreboom A et al (1997) Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A* 94 (5):2031-2035.

367. Stack, Long, and Hawkey (1998) Short- and long-term outcome of patients treated with cyclosporin for severe acute ulcerative colitis. *Alimentary Pharmacology and Therapeutics* 12 (10):973-978.

368. Stahlberg D et al (1996) Pouchitis following pelvic pouch operation for ulcerative colitis. Incidence, cumulative risk, and risk factors. *Dis.Colon Rectum* 39 (9):1012-1018.

369. Staudinger JL et al (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc.Natl.Acad.Sci.U.S.A* 98 (6):3369-3374.

370. Stefansson H et al (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am.J.Hum.Genet.* 71 (4):877-892.

371. Stoll M et al (2004) Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat.Genet.* 36 (5):476-480.

372. Strittmatter WJ et al (1994) Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: implications for Alzheimer disease. *Proc.Natl.Acad.Sci.U.S.A* 91 (23):11183-11186.

373. Strober W, Fuss IJ, and Blumberg RS (2002) The immunology of mucosal models of inflammation. *Annu.Rev.Immunol.* 20:495-549.

374. Sugimura K et al (1993) Analysis of genes within the HLA region affecting susceptibility to ulcerative colitis. *Hum Immunol* 6 (2):112-118.

375. Summers RW et al (2005) Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 128 (4):825-832.

376. Sutherland L and Macdonald JK (2006a) Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane.Database.Syst.Rev.* (2):CD000543.

377. Sutherland L and Macdonald JK (2006b) Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane.Database.Syst.Rev.* (2):CD000544.

378. Sutherland L et al (2002) Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane.Database.Syst.Rev.* (4):CD000544.

379. Swidsinski A et al (2002) Mucosal flora in inflammatory bowel disease. *Gastroenterology* 122 (1):44-54.

380. Tanabe M et al (2001) Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. *J Pharmacol Exp Ther* 297 (3):1137-1143.

381. Tang K et al (2002) Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three ethnic Asian populations. *Pharmacogenetics* 12 (6):437-450.

382. Targan SR et al (2005) Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 128 (7):2020-2028.

383. Thiebaut F et al (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A*84 (21):7735-7738.

384. Thompson NP et al (1996) Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ* 312 (7023):95-96.

385. Toh S et al (1999) Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin-Johnson syndrome. *Am.J.Hum.Genet.* 64 (3):739-746.

386. Torkvist L et al (1999) Low molecular weight heparin as an adjuvant therapy in active ulcerative colitis. *Aliment Pharmacol Ther* 13:1323-1328.

387. Torkvist L et al (2006a) Contribution of CARD15 variants in determining susceptibility to Crohn's disease in Sweden. *Scand.J.Gastroenterol.* 41 (6):700-705.

388. Torkvist L et al (2006b) Contribution of CARD15 variants in determining susceptibility to Crohn's disease in Sweden. *Scand.J.Gastroenterol.* 41 (6):700-705.

389. Torok HP et al (2005) Polymorphisms in the DLG5 and OCTN cation transporter genes in Crohn's disease. *Gut* 54 (10):1421-1427.

390. Tountas NA et al (1999) Functional and ethnic association of allele 2 of the interleukin-1 receptor antagonist gene in ulcerative colitis. *Gastroenterology* 117 (4):806-813.

391. Toyoda H et al (1993) Distinct associations of HLA class II genes with inflammatory bowel disease. *Gastroenterology* 104 (3):741-748.

392. Travis SP (2006) Review article: induction therapy for patients with active ulcerative colitis. *Aliment.Pharmacol.Ther.* 24 Suppl 1:10-16.

393. Travis SP et al (1996) Predicting outcome in severe ulcerative colitis. *Gut* 38 (6):905-910.

394. Trezise AE et al (1992) The multidrug resistance and cystic fibrosis genes have complementary patterns of epithelial expression. *EMBO J* 11 (12):4291-4303.

395. Trinh TT and Rioux JD (2005) Understanding association and causality in the genetic studies of inflammatory bowel disease. *Gastroenterology* 129 (6):2106-2110.

396. Truelove SC and Witts LJ (1955) Cortisone in ulceraticve colitis: Final report on a therapeutic trial. *BMJ* (2):1041-1048.

397. Truelove SC et al (1978) Further experience in the treatment of severe attacks of ulcerative colitis. *Lancet* 2 (8099):1086-1088.

398. Tysk C et al (1988b) Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 29 (7):990-996.

399. Tysk C et al (1988a) Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 29 (7):990-996.

400. Ueda H et al (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423 (6939):506-511.

401. Ueda K et al (1987) The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation. *J Biol Chem* 262 (2):505-508.

402. Ueda K et al (1992) Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem* 267 (34):24248-24252.

403. Urcelay E et al (2006) MDR1 gene: susceptibility in Spanish Crohn's disease and ulcerative colitis patients. *Inflamm.Bowel Dis* 12 (1):33-37.

404. Van Assche G et al (2003) Randomized, double-blind comparison of 4 mg/kg versus 2 mg/kg intravenous cyclosporine in severe ulcerative colitis. *Gastroenterology* 125:1025-1031.

405. Van Assche G et al (2006) Daclizumab, a humanised monoclonal antibody to the interleukin 2 receptor (CD25), for the treatment of moderately to severely active ulcerative colitis: a randomised, double blind, placebo controlled, dose ranging trial. *Gut* 55 (11):1568-1574.

406. Van Assche G et al (2005) Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N.Engl.J Med.* 353 (4):362-368.

407. van Bodegraven AA et al (2006) Genetic Variation in Myosin IXB Is Associated With Ulcerative Colitis. *Gastroenterology* 131 (6):1768-1774.

408. Van Den Heuvel-Eibrink MM et al (2001) MDR1 gene-related clonal selection and P-glycoprotein function and expression in relapsed or refractory acute myeloid leukemia. *Blood* 97 (11):3605-3611.

409. van Deventer SJ et al (2006) A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment.Pharmacol.Ther.* 23 (10):1415-1425.

410. Van Eerdewegh P et al (2002) Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 418 (6896):426-430.

411. van Heel DA et al (2004) Inflammatory bowel disease susceptibility loci defined by genome scan meta-analysis of 1952 affected relative pairs. *Hum.Mol.Genet.* 13 (7):763-770.

412. van Schaardenburg D et al (1995) Prednisone treatment of elderly-onset rheumatoid arthritis. Disease activity and bone mass in comparison with chloroquine treatment. *Arthritis Rheum* 38:823-830.

413. Vecchi M et al (2001) Oral versus combination mesalazine therapy in active ulcerative colitis: a double-blind, double-dummy, randomized multicentre study. *Aliment.Pharmacol.Ther.* 15 (2):251-256.

414. Vermeire S et al (2005) Association of organic cation transporter risk haplotype with perianal penetrating Crohn's disease but not with susceptibility to IBD. *Gastroenterology* 129 (6):1845-1853.

415. Weale ME et al (2003) Selection and Evaluation of Tagging SNPs in the Neuronal-Sodium-Channel Gene SCN1A: Implications for Linkage-Disequilibrium Gene Mapping. *Am J Hum Genet* 73 (3):551-565.

416. Wehkamp J et al (2005) Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc.Natl.Acad.Sci.U.S.A* 102 (50):18129-18134.

417. Westphal K et al (2000) Induction of P-glycoprotein by rifampin increases intestinal secretion of talinolol in human beings: a new type of drug/drug interaction. *Clin Pharmacol Ther* 68 (4):345-355.

418. Wijnholds J et al (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nat Med* 3 (11):1275-1279.

419. Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. *Nat.Rev.Drug Discov.* 1 (4):259-266.

420. Yacyshyn B, Maksymowych W, and Bowen-Yacyshyn MB (1999) Differences in P-glycoprotein-170 expression and activity between Crohn's disease and ulcerative colitis. *Hum Immunol* 60 (8):677-687.

421. Yumoto R et al (2001) Pharmacokinetic interaction of cytochrome P450 3A-related compounds with rhodamine 123, a P-glycoprotein substrate, in rats pretreated with dexamethasone. *Drug Metab Dispos* 29 (2):145-151.

422. Zachos M, Tondeur M, and Griffiths AM (2001) Enteral nutritional therapy for inducing remission of Crohn's disease. *Cochrane.Database.Syst.Rev.* (3):CD000542.

423. Zeggini E et al (2005) An evaluation of HapMap sample size and tagging SNP performance in large-scale empirical and simulated data sets. *Nat.Genet.* 37 (12):1320-1322.

424. Zhang J et al (2001) The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* 11 (7):555-572.

425. Zhang K et al (2004) Haplotype Block Partitioning and Tag SNP Selection Using Genotype Data and Their Applications to Association Studies. *Genome Res.* 14 (5):908-916.

426. Zhao JH, Curtis D, and Sham PC (2000) Model-free analysis and permutation tests for allelic associations. *Hum.Hered.* 50 (2):133-139.

427. Zhao JY et al (1993) Modulation of multidrug resistance gene expression by dexamethasone in cultured hepatoma cells. *Endocrinology* 133 (2):521-528.

428. Zheng H et al (2002) The MDR1 polymorphisms at exons 21 and 26 predict steroid weaning in pediatric heart transplant patients. *Hum.Immunol.* 63 (9):765-770.

429. Zhou C et al (2006b) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J.Clin.Invest* 116 (8):2280-2289.

430. Zhou C et al (2006a) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J.Clin.Invest* 116 (8):2280-2289.